UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

REACTIVE SURFACES LTD., LLP

Petitioner

v.

TOYOTA MOTOR CORPORATION

Patent Owner

CASE: To Be Assigned

Patent No. 8,394,618 B2

DECLARATION OF DR. DAVID ROZZELL IN SUPPORT OF PETITION FOR *INTER PARTES* REVIEW OF U.S. PATENT NO. 8,394,618 B2

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ATTACHMENTS:

I. INTRODUCTION

1. My name is J. David ("David") Rozzell. For more than 2 decades, I have worked in the biotechnology industry with a specialization in the development of new enzymes and their applications. I currently work at Provivi, Inc. as Sr Vice-President of Biocatalysis, directing projects for the development of new enzymes and their use in the production of chemical compounds. I am also a founder and principle with Sustainable Chemistry Solutions, Inc., which is a consulting company and publisher of information products for the enzyme and biocatalysis markets. I am also co-founder and current CEO of Catylix, Inc., a company developing new fluorination chemistry and its applications. Further details of my education, work experience, selected publications, authored books, and patents on which I am an inventor are provided in my resume, which is **Attachment A** to this Declaration.

2. I have been engaged to investigate and opine on certain issues relating to U.S. Patent No. 8,394,618 B2 entitled "LIPASE-CONTAINING POLYMERIC COATINGS FOR THE FACILITATED REMOVAL OF FINGERPRINTS ("the '618 Patent" [Ex. 1001]) in Petition for *Inter Partes* Review of the '618 Patent ("the '618 IPR Petition") which requests the Patent Trial and Appeal Board ("PTAB") to review and cancel Claims 1-11 of the '618 Patent.

3. I understand that, according to USPTO assignment records, the '618 Patent is owned by Toyota Motor Corporation.

4. In this declaration, I will discuss the technology related to the '618 Patent, including an overview of that technology as it was known prior to, and up to the time of the filing of U.S. application 12/820,063 ("the '063 Application) from which the '618 Patent issued. My understanding is that the earliest effective filing date of the'063 Application is June 21, 2010. This overview of the relevant technology provides some of the bases for my opinions with respect to the '618 Patent.

5. This declaration is based on the information currently available to me. To the extent that additional information becomes available, I reserve the right to continue my investigation and study, which may include a review of documents and information that may be produced, as well as testimony from depositions that may not yet be taken.

6. In forming my opinions, I have relied on information and evidence identified in this declaration, including the '618 Patent, the prosecution history of the '618 Patent, and prior art references listed as Exhibits to the Petition for *Inter Partes* Review of the '618 Patent. I have also relied on my own experience and expertise in the relevant technologies and systems that were already in use prior to, and within the timeframe of, the earliest effective filing date of

the claimed subject matter in the '618 Patent (i.e., June 21, 2010).

II. SUMMARY OF OPINIONS

7. The claims of the '618 Patent are directed to technical issues or needs that were recognized and well understood, and technical solutions that were well developed to address the technical issues or needs, at the time of filing the application from which the '618 Patent issued.

8. For purpose of my analysis in this declaration only and based on the disclosure and file history of the '618 Patent, my understanding of certain terms in Claims 1-11 are discussed in detail in a later part of this declaration.

9. In simple terms, Claims 1-11 of the '618 Patent attempt to claim a method of facilitating removal of a fingerprint from a substrate or a coating, which was is an inherent functionality of a substrate or coating having a lipase associated therewith. It is my opinion that the claims of the'618 Patent are rendered obvious by the prior art cited in the '618 IPR Petition in light of such inherent functionality of a lipase that is associated with a substrate or coating because there is nothing novel or non-obvious in such claims and because such claims merely recite routine and common limitations that were well known in the art before the filing date of the application from which the '618 Patent issued.

10. The subsequent sections of this declaration will first provide my qualifications and experience and then describe details of my analysis,

observations and further opinions with respect to the Claims 1-11 of the '618 Patent.

III. QUALIFICATIONS AND EXPERIENCE

A. Education and Work Experience

11. I obtained a Bachelor of Science degree in Chemistry in 1978 from the University of Virginia and a Ph.D. in Chemistry from Harvard University in 1983. My dissertation was entitled: "Stereospecificity of Acetoacetate Decarboxylase. A New Synthesis of Chiral Methyl Acetate."

12. I have authored dozens of peer reviewed journal articles, several chapters in books, and given numerous presentations at symposia around the world in the field of enzymes, biocatalysis, and organic chemistry. (See relevant sections of **Attachment A)**.

13. I currently serve as Sr. Vice-President, Biocatalysis, at Provivi, Inc. in Santa Monica, CA. I joined Provivi, Inc. in 2015 with the responsibility of leading development and commercialization of novel enzymes catalyzing the synthesis of chiral cyclopropanes via a carbene transfer mechanism. My specific responsibilities include managing internal R & D, business development, customer acquisition and project management to meet rigorous timelines for development.

14. I currently also serve as CEO and Founder of Sustainable Chemistry Solutions, Inc., in Burbank, CA. Through Sustainable Chemistry Solutions, Inc., I am the publisher of the web site http://www.bio-catalyst.com, which provides information and insights on biofuels, bio-based chemicals, and biocatalysis and am also the publisher of monthly newsletter *Enzyme Industry Newsletter,* offering information products and consulting services related to enzymes and biocatalysis to pharmaceutical and chemical companies. I provide consulting support to programs for the development of novel enzymes and their applications, and also for pathways in bio-based chemical production. I am creator and publisher of the Enzyme Company Guide and the Biocatalysis Company Guide, providing business and technical information to industry specialists. I also offer expert witness services in patent litigation and cases involving enzymes and the development and enzyme-based processes and applications.

15. Since 2011, I have served as CEO and Co-Founder of Catylix, Inc. in Burbank, CA. Together with the other co-founder, we established this company to develop and commercialize a novel, broadly-useful chemistry for adding fluorine-containing functional groups to chemical compounds. Our first product called Trifluoromethylator® was launched in July 2011. The

main product applications are in the discovery of pharmaceuticals and crop protection agents with improved efficacy and metabolic stability.

16. I served as President and CEO of Solidus Biosciences, Inc. in San Francisco, CA from 2009 to 2010. In leading this company, which was developing a novel, chip-based *in vitro* toxicology platform, I was responsible for managing company operations, setting business strategy, developing new customer relationships, and raising funds from investors.

17. From 2007 to 2008, I served as VP, Biocatalysis Technology and Applications for Codexis, Inc. following its acquisition of BioCatalytics, Inc. in July 2007. I was responsible for the identification and development of new technologies, including technologies developed and in-licensed through external collaborations. I managed a network of external collaborations in the USA and Europe, promoting the company and supporting business development activities through technical presentations, press conferences, and written articles. I also initiated an emphasis on Green Chemistry.

18. I was Founder, President, CSO and CEO at BioCatalytics, Inc. in Pasadena, CA from 1996 to 2007. I established this biotechnology company to develop and commercialize enzymes and enzyme-based processes for the production of optically active pharmaceutical intermediates and other specialty chemicals. I built this company into a profitable seller of novel enzymes for

chemical synthesis, with one of the world's largest enzyme product lines. I established a European office in 2005 and a subsidiary BioCatalytics Europe GmbH in Graz, Austria in 2006. BioCatalytics, Inc. was acquired in 2007 by Codexis, Inc.

19. I was Co-Founder and Acting CEO of EraGen Biosciences, Inc. (initially established as Sulfonics, Inc.) in Madison, WI from 1994-1996. I co-founded this start-up biotechnology company, which focused on applications of non-standard nucleic acid bases and protein structure prediction. This company raised seed capital from individual investors and the Novartis Venture Fund. I acted as CEO until a full-time person was recruited to establish the company in its first headquarters in Florida.

20. During my time with Exogene Corporation in Monrovia, CA, I first served as Vice-President of Research & Development (1991-1992) and then as President (1992-1994). My responsibilities included business development, negotiation of sponsored research and technology licensing agreements, general scientific guidance of the company's research, and supervision of the administrative and senior scientific staff.

21. I served as Director of Research and Biotreatment Systems at Celgene Corporation in Warren, NJ from 1988-1991. This company employed a combination of biocatalytic reactions and organic chemistry. My

responsibilities included directing both proprietary and collaborative research programs focused on the production of pharmaceutical intermediates and specialty chemicals and in the biocatalytic degradation of environmentally-hazardous chemicals in waste streams.

22. During my time with Genetics Institute, Inc. in Cambridge, MA, I first served as Senior Scientist (1983-1986) and then as Director of Biocatalysis Research (1986-1988). I built and managed an interdisciplinary group of professionals and directed the research and development activities of an Applied Enzymology group and a Biocatalysis group. My efforts in these positions resulted in more than $1 million in revenues through funding and license agreements, and the commercialization of processes to manufacture optically active amino acids at the multi-hundred ton per year scale.

23. With respect to the claimed invention, I have specific experience in the areas of the cloning and expression of genes encoding enzymes, the improvement of enzymes through directed evolution methods, the use of enzymes to catalyze various chemical reactions, and the immobilization of enzymes on polymeric materials and surfaces by either covalent or non-covalent means. In my previous research work, I have immobilized various types of enzymes, including lipases, proteases, amidases, esterases, oxidoreductases, transaminases, and other enzymes on various types of materials. This work

includes specific examples of immobilization by both covalent (chemical bonding) and non-covalent (adsorption, entrapment in a polymeric gel or coating) methods. In one case, I adsorbed lipases from different sources onto cross-linked polystyrene and polyacrylate-co-polymers for use in hydrolyzing or transesterifying esters. I have also immobilized enzymes by entrapment in gels formed by the condensation of polymers such as chitosan calcium alginate, and kappa-carrageenan, or by entrapment within gels formed by a polymerization or curing process, such as the polymerization of polyacrylamide. I have also immobilized enzymes onto materials such as silica or alumina which have had their surfaces chemically modified or coated with an organic compound or polymer.

24. I have also published articles about enzyme immobilization. I was a co-author of a book chapter describing methods of covalent enzyme immobilization entitled "*Immobilization of Enzymes by Covalent Attachment.*" This was published as chapter 20 in "Methods in Biotechnology, Vol. 17: Microbial Enzymes and Biotransformations," edited by J. L. Barredo and published by Humana Press, Inc. Totowa, NJ. (**Attachment B**) I am also the author of "*Immobilization of Enzymes: Techniques and Applications*" published as Chapter 13 in the book "Biocatalytic Production of Amino Acids and Derivatives: New Developments and Process Considerations," published by

Hanser Publishers in 1992 (**Attachment C**). I also have written about my research on the use of immobilized Transaminases for the production of amino acids [see, for example, "*Immobilized Aminotransferases for Amino Acid Production*": J. David Rozzell., "Methods in Enzymology" Volume 136, Pages 479-497, (1987) Immobilized Enzymes and Cells, Part C, ISBN:978-0-12-182036-7.] (**Attachment D**).

B. Compensation

25. My services in this matter, which are being provided through Sustainable Chemistry Solutions, Inc., whose offices are located at 437 South Sparks Street, Burbank, California 91506, are being compensated at a rate of $375 per hour. This compensation is not contingent upon my performance, the outcome of this *inter partes* review or any other proceeding, or any issues involved in or related to this *inter partes* review.

C. Documents and Other Materials Relied Upon

26. The documents on which I rely for the opinions expressed in this declaration are documents and materials identified in this declaration, including the '618 Patent, any related patents and applications in the same family as the '618 Patent, the prosecution history for the '618 Patent and that of any related family members of the '618 Patent, the cited prior art references and associated information discussed in this declaration, and any other references

specifically identified in this declaration, in their entirety, even if only portions of these documents are discussed here in an exemplary fashion.

IV. STATEMENT OF LEGAL PRINCIPLES

A. Claim Construction

27. I understand that, when construing claim terms, a claim subject to *inter partes* review receives the "broadest reasonable construction in light of the specification of the patent in which it appears." I further understand that the broadest reasonable construction is the broadest reasonable interpretation ("BRI") of the claim language, and that any term that lacks a definition in the specification is also given a broad interpretation.

B. Anticipation

28. I understand that in order for a patent claim to be valid, the claimed invention must be novel. I further understand that if each and every element of a claim is disclosed in a single prior art reference, then the claimed invention is anticipated, and the invention is not patentable according to pre-AIA 35 U.S.C. § 102 effective before March 16, 2013. I also understand, in order for the invention to be anticipated, each element of the claimed invention must be described or embodied, either expressly or inherently, in the single prior art reference. It is also my understanding that, in order for a reference to inherently disclose a claim limitation, that claim limitation must necessarily

be present in the reference. I also understand that a prior art reference must be enabling in order to anticipate a patent claim.

C. Obviousness

29. I understand that obviousness under pre-AIA 35 U.S.C. § 103 effective before March 16, 2013 is a basis for invalidity. Specifically, I understand that where a prior art reference discloses less than all of the limitations of a given patent claim, that patent claim is invalid if the differences between the claimed subject matter and the prior art reference are such that the claimed subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the relevant art ("POSITA"). It is also my understanding that obviousness can be based on a single prior art reference or a combination of references that either expressly or inherently disclose all limitations of the claimed invention.

V. LEVEL OF ORDINARY SKILL IN THE ART

30. I understand that the claims and specification of a patent must be read and construed through the eyes of a POSITA at the time of the earliest effective filing date of the application from which the patent issued. I also understand that to determine the appropriate level of a POSITA, the following factors may be considered: (a) the types of problems encountered by those working in the field and prior art solutions thereto; (b) the sophistication of the

technology in question, and the rapidity with which innovations occur in the field; (c) the educational level of active workers in the field; and (d) the educational level of the inventor.

31. Based on the above considerations and factors, it is my opinion that a person having ordinary skill in the art would have at least a bachelor's degree plus 5 or more years of experience, or a Masters or PhD degree with 2 or more years of experience in chemistry, biochemistry, molecular biology, biochemical engineering, or a related discipline. This description is approximate and additional years of experience can compensate for less formal education in a discipline, and a POSITA could have combined experience in more than one of the disciplines listed above.

VI. TECHNOLOGY BACKGROUND OF CLAIMED SUBJECT MATTER OF THE '618 PATENT

A. Disappearance of Fingerprints by Vaporization Occurs Without Enzyme

32. As an initial matter, it is important to understand that it is well-established that fingerprints disappear from surfaces over time without having been contacted with an enzyme such as a lipase, and that the mechanism of this disappearance is by vaporization.

33. Buchanan [Ex. 1013] studied fingerprints from about 50 individuals ranging in age from 3 to 64 years, the fingerprints were analyzed for their

compositions by gas chromatography to characterize, in particular, the volatility of the components. In general, the author found that samples obtained from children contained more volatile fatty acids, esters, and related compounds, and samples from adults contained less volatile fatty acids, esters, and related compounds, providing a differentiating characteristic between the fingerprints of adults and children that could be useful in solving crimes. (*Id.* at Abstract:1-8) In a real-world test of these laboratory results, the author further found that fingerprints deposited by children in vehicles disappeared in less than 24 hours; fingerprints from adults lasted at least several days. No treatments of any kind were applied to the fingerprints to facilitate their disappearance. The author further found that when fingerprints were subjected to mild heating (allowing the vehicle to sit in sunlight in the summer), the rate of their disappearance was faster, (*Id.* at 89:21-29) as would be expected from a vaporization mechanism in my opinion. Buchanan thus concluded that fingerprints and their components can disappear by vaporization, and that this vaporization would occur under ambient conditions or with mild heating, and that fingerprints containing more volatile components vaporized more quickly than fingerprints containing a lower amount of volatile components. (*Id.* at 91:1-7)

34. Given that Buchanan demonstrates that a fingerprint or a similar mark or stain

on a surface will become less visually apparent over time, and that the mechanism of its disappearance is by vaporization, it is my opinion that it would be obvious to a POSITA that fingerprints can be removed from a surface, substrate or coating by vaporization, and that the disappearance by vaporization would occur whether or not the fingerprint has been in contact with an enzyme such as a lipase. In my opinion it is an inherent property of fingerprints that they would disappear from a surface by vaporization. Reading of Buchanan (e.g., *Id.* at 89:21-29; 91:1-7) would make it further obvious to a POSITA that fingerprints containing more volatile (i.e. lower molecular weight) components would disappear by vaporization more quickly.

B. Inherent Enzymatic Functionality of Lipase is to Hydrolyze Lipids and Esters

35. It is well-known that lipases in general will hydrolyze, and thereby degrade, various lipid-based compounds. Lipases will degrade triglycerides and other lipids, wax esters, other fatty acid esters, cholesterol esters, and similar compounds, which are well-known to be among the components of fingerprints (see, for example, Buchanan at Abstract:1-8; 90:31 to 91:19). In fact, the '618 Patent states that these types of compounds are well-known to be present in fingerprints and similar stains ('618 Patent at 2:38-48), and that

lipases are well-known to degrade these types of compounds (*Id.* at 2:34-37, 2:43-48). Indeed, hydrolyzing various types of lipids and fatty acid esters is a defining characteristic of a lipase and an inherent property of lipases in general. For example, the products of a triacylglycerol lipase's enzymatic action on a triacylglycerol lipid as would be found in a fingerprint produces glycerols and fatty acids. [See , **Attachment E**; Enzyme Nomenclature 1978, published in 1979, Academic Press, New York, pp. 234-239].

36. Further, a lipase non-covalently immobilized on a surface has also been described, in which the lipase was absorbed onto a fabric surface to facilitate removal of an oil stain (See **Attachment F**, U.S. Patent 6,265,191, at 6: 5-8:50). In my opinion, it would be an inherent property that a lipase immobilized on a surface would degrade a triglyceride-based oil stain.

37. Therefore, in my opinion, it is unsurprising and completely expected that a lipase would degrade lipid and ester components of a fingerprint, and therefore the degradation of fingerprint components by a lipase would have been obvious to a POSITA at the time of the invention.

C. Enzymatic Degradation of Components of a Fingerprint by a Lipase Immobilized in or on a Substrate or Coating

38. The materials described in the '618 patent are various substrates or coatings that have a lipase enzyme adsorbed, covalently attached, or otherwise

adhered to or entrapped in that substrate or coating. (*Id.* at 7:26-39, 8:13-18, 8:39-46, 15:30-32) Such materials are well-known in the art. (See Sections VI(E) and (F) in this declaration for a more detailed explanation.) It has also been shown in many cases and with many different materials, including substrates and coatings such as those described in the '618 patent, that catalytic activity of the lipase is typically retained on immobilization in or on the substrate or coating. As such, the substrates or coatings with lipases attached or adhered thereto as described in the '618 patent are similar, if not identical, to numerous other immobilized lipase materials that have been prepared and described in the prior art. Juxtaposing the description of lipases immobilized in or on a surface, polymer, substrate, or coating as provided in the '618 patent with the vast amount of prior art describing lipases immobilized in or on similar surfaces, polymers, substrates, or coatings [See, for example, **Attachment G**; 1. "*Optical resolution of dl-menthol by entrapped biocatalysts*": Saburo Fukui and Atsuo Tanaka, "Methods in Enzymology" Volume 136, Pages 293-302, (1987) Immobilized Enzymes and Cells, Part C, ISBN:978-0-12-182036-7.; 2. "*Industrial operation of immobilized enzymes*": M.J. Daniels, "Methods in Enzymology" Volume 136, Pages 371-379, (1987) Immobilized Enzymes and Cells, Part C, ISBN:978-0-12-182036-7.; 3. "*Regiospecific interesterification of*

triglyceride with celite-adsorbed lipase": Shigeru Yamanaka and Takashi Tanaka, "Methods in Enzymology" Volume 136, Pages 405-411, (1987) Immobilized Enzymes and Cells, Part C, ISBN:978-0-12-182036-7.; and 4. "*Production of aspartame by immobilized thermoase*": Kiyotaka Oyama, Shigeaki Irino and Norio Hagi, "Methods in Enzymology" Volume 136, Pages 503-516, (1987) Immobilized Enzymes and Cells, Part C, ISBN:978-0-12-182036-7"], we have an direct comparison of lipase-associated materials and their functionality.

39. Because a lipase immobilized on or within a surface, polymer, substrate, or coating would be expected to retain at least some of its inherent activity to hydrolyze various ester and lipid components such as those components found in a fingerprint, in my opinion it would have been obvious to a POSITA at the time of the invention to employ a lipase immobilized in or on a coating as described in the '618 patent for the purpose of degrading one or more lipid components of a fingerprint.

D. Degradation of Components of a Fingerprint by a Lipase Makes the Components More Volatile and More Easily Vaporized

40. Buchanan described the analysis of fingerprints of adults and children by gas chromatography, showing that the fingerprints of children have more volatile components than the fingerprints of adults, and are therefore more readily

vaporizable and disappear more quickly. (Buchanan at Abstract:1-8, 89:21-29; 91:1-7). Thus, one or more of the components of fingerprints will disappear without the presence of a lipase associated substrate or coating as described in the '618 patent.

41. Lipase-catalyzed degradation of lipid substances, such as those known to be components of fingerprints, break these components down into compounds of lower molecular weight and generally higher volatility. Thus, the inherent activity of a lipase to break down lipid components of fingerprints into more volatile substances will clearly enhance the removal of those substances by vaporization. Therefore, in my opinion, it would have been obvious to a POSITA at the time of the claimed invention of the '618 Patent to employ a lipase in or on a surface or coating to degrade lipid and ester components of a fingerprint, rendering them more volatile and more easily vaporized, and thereby facilitating the removal of the fingerprint.

E. Covalent Attachment to a Substrate or Coating Was Well-Known at the Time of the Claimed Invention

42. Methods of attaching enzymes to a substrate or coating are well-known and have been in use for at least 30 years. These methods include attachment of enzymes to surfaces or coatings by covalent methods. Examples of enzymes immobilized by these covalent methods include enzymes such as lipases,

amylases, proteases, and other enzymes. The covalent methods referenced in the '618 patent ('618 Patent at 7:26-8:13) are in publications dating from the 1980s (see, for example, **Attachment G**), and these methods are all well-known in the prior art. No new methods for covalent immobilization were provided. Therefore, it is my opinion that immobilizing a lipase in a coating by covalent methods as described in the '618 patent would have been well-known at the time of the claimed invention and would have been obvious to a POSITA.

F. Non-Covalent Adherence to a Substrate or Coating Was Well-Known at the Time of the Claimed Invention

43. Methods of attaching enzymes to a substrate or coating by non-covalent methods are also well-known and have been in the prior art for more than 25 years (see, for example, **Attachment G**). Examples of these non-covalent methods include adsorption on a surface, entrapment in a gel or resin that can be cured or cross-linked. Non-covalent immobilization of lipases, amylases, proteases, and other enzymes have all been described. The non-covalent methods referenced in the '618 patent (e.g., the '618 Patent at 7:26-39, 8:13-18, 8:39-46, 15:30-32) are thus well-known in the prior art (see, for example, **Attachment G**). No new methods for non-covalent immobilization were provided. Therefore, it is my opinion that immobilizing a lipase in a coating

by non-covalent methods as described in the '618 patent would have been well-known at the time of the claimed invention and would have been obvious to a POSITA.

G. The Selected Lipase Species Are Well-Known and Not Novel

44. A large number of lipases have been identified and described, often with characterization of the ability of these enzymes to hydrolyze lipids, fatty acid esters, and similar ester substrates. These lipases are known by various other names, including acyl glycerol lipase, triacylglycerol lipase, lipoprotein lipase, phospholipase A1, phospholipase A2, phospholipase C, phospholipase D, cholesterol ester hydrolase, and a number of others. While each of these lipases may have a different range of esters that it will act on, and often these ranges of esters will overlap, the range of lipases recited in the '618 Patent (e.g., *Id.* at 3:9-14) are all well-known in the prior art. (See, for example, **Attachment E**). Thus, in my opinion, it would have been obvious to a POSITA to select a lipase from any of the known lipases to hydrolyze and degrade lipid components of a fingerprint.

H. Many Polymeric Substrate/Coatings Have Been Used for Enzyme Immobilization or Association

45. Many different substrates, polymers, surfaces, and coatings have been used for the immobilization of enzymes. These surfaces, polymers, substrates and

coatings include inorganic surfaces such as silica or alumina, organic polymers such as poly-methyl methacrylate, styrene-divinyl benzene co-polymers, polyurethanes, chitosan, and many others. Lipases and other enzymes have also been entrapped in polymers, or adsorbed on polymeric surfaces. Immobilizing, entrapping, or otherwise associating a lipase with a surface, polymer, substrate, or coating was well-known at the time of the '618 invention (see, for example, **Attachment G**), as is acknowledged in the '618 Patent itself (see the '618 Patent, 8:7-12).

VII. OVERVIEW OF THE '618 PATENT

46. The invention of the '618 Patent is not restricted to any particular intended applications or products. In this regard, it is disclosed in the '618 Patent that, "The following description of embodiment(s) of the invention is merely exemplary in nature and is in no way intended to limit the scope of the invention, its application, or uses, which may, of course, vary." (the '618 Patent [Ex. 1001] at 2:25-28). Moreover, there is no element or limitation in that any off the claims that would necessarily limit the claimed invention to a particular application or product. To the contrary, the claimed invention of the '618 Patent reads on a broad collection of applications and products, including but not limited to consumer applications and products, medical applications and products, industrial application and products, etc. Specific examples of

such products include, but are not limited to, automobiles, medical devices and supplies, electronic devices, eyewear, and any other devices and articles that can come into contact with a bioorganic stain that is capable of being enzymatically degraded by a lipase.

47. The '618 Patent discloses, "a composition and method for fingerprint removal from a substrate surface is disclosed. The method includes associating a lipase with a substrate or a coating such that the lipase is capable of enzymatically degrading a component of a fingerprint." (*Id.* at 1:47-51). "The composition includes a substrate or coating containing a lipase. The composition optionally includes an organic crosslinkable or non-crosslinkable polymer resin." (*Id.* at 1:59-61).

48. The '618 Patent discloses that "a fingerprint as defined herein is a bioorganic stain, mark, or residue left behind after an organism touches a substrate or coating. A fingerprint is not limited to marks or residue left behind after a substrate is touched by a finger. Other sources of bioorganic stains are illustratively, palms, toes, feet, face, any other skin surface area, hair, stains from fats used in cooking such as cis-fatty acids, or fatty acids from any other source." (*Id.* at 3:1-8).

49. The present invention of the '618 Patent is based on "the catalytic activity of a lipase enzyme to selectively degrade and volatilize components of

fingerprints, thus, promoting active fingerprint removal. Fingerprint stains typically include components of sweat gland secretion and sebum which includes lipids, wax, and cellular debris. Several of the substances of sebum are lipophilic and have low volatility such as squalene and wax esters." (*Id.* at 2:34-42). "The lipase that is either immobilized in coatings or substrates catalyzes the hydrolysis, esterification, or transesterification of lipids including triacylglycerols, cholesterol esters, and other fingerprint components into smaller molecules. The smaller molecules may have higher volatility than their precursors and more easily vaporize at ambient or elevated temperatures thereby allowing for complete stain removal." (*Id.* at 2:43-50).

50. The '618 Patent disclosed, "When a surface which is optionally a substrate or a coated substrate, is contacted with a fingerprint, the lipase enzyme or combinations of enzymes contact the fingerprint, or components thereof. The contacting allows the enzymatic activity of the substrate or coating to interact with and enzymatically alter the components of the fingerprint improving their removal from the substrate or coating. It is appreciated that the inventive methods of facilitating fingerprint removal will function at any temperature whereby the lipase is active." (*Id.* at 10:36-45). "The presence of lipase combined with the material of a substrate or a coating on

a substrate, optionally, with applied heat, breaks down fingerprint stains for facilitated fingerprint removal." (*Id.* at 11:4-7).

51. The '618 Patent discloses, "an inventive method uses an inventive composition that is one or more lipases incorporated into a substrate itself or into a coating on the substrate. The lipase enzyme is optionally non-covalently associated and/or covalently attached to the substrate or coating material or is otherwise associated therewith such as by bonding to the surface or by intermixing with the substrate/coating material during manufacture such as to produce entrapped lipase." (*Id.* at 7:26-33). "Lipases are optionally uniformly dispersed throughout the substrate network to create a substantially homogenous protein platform." (*Id.* at 7:58-60). "Lipases are optionally present in a coating that is layered upon a substrate wherein the lipase is optionally entrapped in the coating material, admixed therewith, modified and integrated into the coating material or layered upon a coating similar to the mechanisms described for interactions between a lipase and substrate material." (*Id.* at 8:13-18).

52. The claims of the '618 Patent (i.e., claims 1-11) that are challenged in the '618 IPR Petition recite a method of facilitating the removal of a fingerprint on a substrate or a coating.

VIII. SUMARY OF THE CITED PRIOR ART

1. **Drevon** ([Ex. 1003] Printed Publication; published December 3, 2002)

53. Drevon describes "strategies to immobilize enzymes into various polymer and coatings. Three categories of bioplastic matrices were investigated. The first type of bioplastics was prepared by irreversibly incorporating di-isopropylfluorophosphatase (DFPase) into polyurethane (PU) foams." (*Id.* at 3:5-8). "Biopolymers were also prepared via atom transfer radical polymerization (ATRP) using acrylic and sulfonate-derived monomers. ATRP ensured the covalent and multi-point immobilization of enzyme within polymer matrices." (*Id.* at 3:15-17). "Enzyme-containing PU- and Michael adduct (MA)-based coatings correspond to the last category of bioplastics that was investigated. DFPase was irreversibly incorporated into PU coatings." (*Id.* at 4:1-3).

54. With reference to attaching enzymes to support solid supports, Drevon discloses that "Immobilization refers to the preparation of insoluble biocatalytic derivatives and involves the coupling of enzymes to solid supports that are either organic or inorganic. It has been increasingly used in industrial applications as it facilitates the separation of biocatalysts from the effluents and, hence, the recovery and purification of the products. Moreover, solid biocatalysts offer the major advantage of being reusable. The large variety of matrices that can be used ranges from natural and synthetic

polymers to silica beads. Covalent immobilization often proceeds by the reaction of specific functionalities at the support surface with amino acid side chains that are readily available on the enzyme surface. The covalent coupling may induce drastic changes in the enzymatic kinetics especially when it occurs near the active site. Another important effect is to reduce the enzyme flexibility. As the number of linkages between the enzyme and the support increases, so does the enzyme rigidity. By providing a maximum rigidity, multi-point covalent immobilization is likely to prevent enzyme unfolding upon heating or in the presence of a denaturant. A non-conventional strategy to achieve multi-point covalent immobilization within a polymer network is by copolymerizing the enzyme with monomers capable of a chemical reaction with specific functionalities on its surface. During polymerization, the enzyme acts as a monomer and is, thus, expected to be uniformly distributed within the resulting biopolymer." (*Id.* at 18:7-19:4).

55. It is disclosed in Drevon that "The overall effect of the immobilization process on the enzyme kinetics is given by the activity retention, which corresponds to the ratio of the immobilized and native specificity constants (k_{cat}/K_M). The activity retention of biocatalytic coatings can significantly fluctuate depending on the enzyme and the polymer properties such as crosslinkage and hydrophilicity. The lowest activity retention was observed

for the entrapment of flavin reductase into pyrrole-based coating (0.13 % AR), whereas lipase-containing poly(propylene glycol)-based coating exhibited the highest activity retention (81.6 % AR) (Table 1). The process of immobilization into coatings resulted in most cases in the enhancement of enzyme thermostability (Table 2). As the immobilized enzyme is locked within the polymeric matrix, it is less flexible et hence less susceptible to denaturation." (*Id.* at 79:7-17).

56. Drevon discloses that "waterborne polyurethane (PU) coatings result from the polymerization of aqueous polyester-based polyol dispersions and water dispersible aliphatic polyisocyanates. As the film is cured at room temperature, water evaporates and cross-linking occurs through the condensation between hydroxyl groups and isocyanate functionalities (Figure 10). Cross-linking provides water resistance to the coatings. Two-component waterborne polyurethanes are increasingly used in industrial applications, and they exhibit properties similar to those of solvent borne polyurethane coatings. Waterborne polyurethane coating represents a potentially ideal polymeric matrix for multipoint and covalent immobilization of enzymes. Given our depth of understanding of monolith polyurethane-enzyme composites, we believe that an enzyme added to the aqueous phase of a two-component system prior to polymerization can act as a monomer during

coating curing. The immobilization process would rely on the ability of amines at the enzyme surface to react with isocyanate functionalities at a faster rate than hydroxyl groups on the polyol (Figure 10). A similar approach has been used for the insertion of enzyme into hydrophobic acrylate polymer coatings." (*Id.* at 101:2-17).

57. With respect to waterborne PU coatings, Drevon further discloses "Two-component waterborne PU coatings are progressively replacing solvent borne PU coatings for a large range of applications. The coating curing occurs at room temperature and involves the reaction of hydroxyl groups on a polyol dispersion with isocyanates functionalities of a water-dispersible polyisocyanate. The resulting films are highly crosslinked and water-resistant. Given their water-based chemistry, PU coatings constitute a potential matrix for enzyme immobilization. The enzyme can be solubilized in the polyol water dispersion. Once contacted with the polyisocyanate prepolymer the primary amines at the enzyme surface react with isocyanate functionalities at a faster rate than hydroxyl groups on the polyol." (*Id.* at 106:5-13).

2. <u>Schneider</u> ([Ex. 1004] U.S. Appl. Pub. No. 2005/0147579 A1; published July 7, 2005)

58. Schneider discloses, "a coating composition comprising at least one

enzyme capable of acting on a compound, wherein said action results in the formation of an antifouling species comprising an antifouling activity, and wherein said compound does not form part of said coating composition. The coating composition preferably comprises at least one oxidase capable of acting on a compound, such as a substrate for said oxidase, wherein said action results in the formation of an antifouling species including an antimicrobial species comprising an antimicrobial activity. More preferred, the oxidase comprises an activity which results in the formation of a peroxide. The oxidase can be present in said coating composition in combination with one or more additional enzymes including, but not limited to, an esterase, including a lipase, an amidase, including a protease, and a polysaccharide degrading enzyme, wherein said one or more additional enzyme(s), alone or in any combination, can be included in the presence or absence of one or more substrates for one or more of said enzymes." (*Id.* at Abstract:1-20).

59. With respect to antimicrobial activity, Schneider discloses, "As the technology for keeping the interior environment of hospitals, etc., against bacteria and fungi, it is common practice to apply a coating containing a compound having antibacterial/antifungul activity to the surface of the interior walls, fixtures, furnishings, upholstery, etc." (*Id.* at 0009:1-5) and

"The coating compositions of the invention are capable of reducing and/or eliminating fouling in the form of microbial growth and/or the formation of bio-film on objects coated with the composition. The microbial organisms can be e.g. bacteria, vira, fungal cells and slime molds." (*Id.* at 0125:1-5).

3. **Van Antwerp** ([Ex. 1005] U.S. Patent No. 5,868,720; published February 9, 1999)

60. Van Antwerp discloses, "An improved indwelling catheter adapted for long-term usage includes a stable enzyme coating to prevent occlusion of the catheter lumen. The enzyme coating includes a fibrinolytic and/or lipolytic enzyme incorporated in a catheter coating to resist or control proteolytic degradation, thereby maintaining the enzyme in an active state for dissolving clots and occlusions within the catheter lumen over an extended period of time." (*Id.* at Abstract 1-8). "The catheter 10 includes a stable, substantially immobilized enzyme-containing coating 14 as depicted, for example, in FIG. 6, for preventing and/or dissolving occlusions." (*Id.* at 3:44-47). "[T]he catheter 10 is commonly constructed from a polymeric material, such as medical grade silicone rubber, polyethylene, or the like." (*Id.* at 3:65-67). "Alternatively, a lipolytic enzyme such as phospholipase may be used for dissolving a lipid-based occlusion. A combination of such fibrinolytic and lipolytic enzymes may also be used."

(*Id.* at 4:18-21).

61. As shown and discussed in reference to FIGS. 3 and 4 of Van Antwerp, a catheter having an enzyme composition coating on its interior and exterior surfaces is provided. To this end, Van Antwerp discloses, "FIG. 3 illustrates immersion of catheter 10 into a prepared enzyme slurry or emulsion 20. In this regard, the enzyme is commonly available in particulate form, having a particle size ranging on the order of one to fifteen microns. The enzyme particles are mixed in a liquid carrier such as water to produce the emulsion 20 shown in FIG. 3. Upon withdrawal of the catheter 10from the enzyme emulsion 20, the catheter surface is allowed to dry resulting in adherence of the enzyme to the catheter in a micellar array of microsphere particles 21, as shown in exaggerated form in FIG. 4." (*Id.* at 4:36-46).

4. **Moon** ([Ex. 1006] U.S. Appl. Pub. No. 2005/0176905 A1; published August 11, 2005)

62. Moon discloses, "an antimicrobial polymeric resin composition in which one or more antimicrobial polymers selected from the group consisting of the antimicrobial monomer compound of formula 1, the antimicrobial homopolymer of formula 4, the antimicrobial copolymer compound of formula 5, the compounds of formula 6 to 9, and the acrylic copolymer of formula 10 are uniformly mixed with an ordinary polymeric resin." (*Id.* at

0115:1-8). "The acrylic copolymer of formula 10 of the present invention can be manufactured by the radical reaction of the compound of formula 11 with the compound of formula 12 below," (*Id.* at 0109:1-4). "It is preferred that the compound of formula 12 has an acrylic monomer, which is the hydrocarbon chain attributed to an acrylic acid or methacrylic acid, as a main chain. More preferably, it is a monomer having an acrylic acid, acrylic acid alkyl ester, methacrylic acid, or methacrylic acid alkyl ester. The acrylic acid alkyl ester and methacrylic acid alkyl ester preferably include a C_1~ C_{18} alkyl. Examples of such acrylic acid alkyl ester include methylacrylate, ethylacrylate, n-propylacrylate, isopropylacrylate, cyclohexylacrylate, t-butylcyclohexylacrylate, stearylacrylate, and laurylacrylate. Also, the acrylic monomer can comprise a reactive functional group, and as examples of such functional groups, there are an amide group, a hydroxyl group, an epoxy group, a silanol group, and an aldehyde group." (*Id.* at 0112:1-14). With respect to polymer resin composition in accordance with the invention, Moon discloses, "They are particularly useful for medical supplies, that is, medical devices/products for insertion into the human body such as catheters for medical purposes, prostheses, and products for repairing bones, or blood transfusion bags for medical purposes." (*Id.* at 0059:12-16).

5. **Hamade** ([Ex. 1007] U.S. Patent No. 6150146; published November 21, 2000)

63. Hamade discloses, "a novel method for sustained release of compounds having antimicrobial activity and a coating composition capable of releasing a safe and effective compound having antimicrobial activity at a controlled rate." (*Id.* at 0002:49-53). "[T]he coating composition according to the present invention comprises a film-forming resin, an enzyme, and a substrate, said enzyme being capable of reacting with said substrate to produce a compound having antimicrobial activity." (*Id.* at 0007:31-35). With respect to enzyme selection, Hamade discloses, "There is no particular limitation on an enzyme-substrate combination capable of producing such a carboxyl group-containing compound. Typical are the case in which the enzyme is an esterase and the substrate is an ester bond-containing compound and the case in which the enzyme is an amidase and the substrate is an amide bond-containing compound. The esterase is not particularly restricted in kind but includes esterases such as carboxylesterase, arylesterase, acetylesterase, etc.; lipases such as triacylglycerol lipase, lipoprotein lipase, etc.; and proteases such as subtilisin, chymotrypsin, tripsin, elastase, cathepsin, papain, chymopapain, pepsin, etc., and so forth." (*Id.* at 0004:5-18).

6. **McDaniel** ([Ex. 1008] U.S. Appl. Pub. No. 2004/0109853, published June 10, 2004)

64. McDaniel is directed to compositions and methods for their use as components of surface treatments such as coatings. McDaniel discloses, "compositions and methods for incorporating biological molecules into coatings in a manner to retain biological activity conferred by such biological molecule." (*Id.*at 0021:4-6). Such compositions comprise "a bioactive molecule such as an enzyme composition that retains activity after being admixed with paint. In addition, it still retains activity after the paint is applied to a surface, and renders the surface bioactive." (*Id.* at 0023:2-6). "In some embodiments, the coating comprises a paint. In other embodiments, the coating comprises a clear coating. In some aspects, the clear coating comprises a lacquer, a varnish, a shellac, a stain, a water repellent coating, or a combination thereof. In general aspects, the coating comprises a binder, a liquid component, a colorant, an additive, or a combination thereof." (*Id.* at 0046:1-7).

7. **Bostek** ([Ex. 1009] Printed Publication; published December 1992; American Association of Nurse Anesthetists Journal, (60(6):561-6)

65. Bostek discloses, infusing intravenous ("IV") fluid that is heated above room temperature into a patient through a catheter and that such infusing can be for a period of at least 2 hours. (*Id.* at pg. 564, col. 1, ln. 13-16; pg. 564, col. 2, ln.

1-10; pg. 564, col. 2, ln. 14-pg. 565, ln. 12).

IX. SUMMARY OF UNPATENTABILITY OPINIONS

66. I understand that the earliest effective filing date of the '063 Application, from which the '618 Patent issued, is June 21, 2010. As explained below, it is my opinion that the following prior art references, which are listed as Exhibits to the Petition for *Inter Partes* Review of the '618 Patent, disclose all elements and limitations recited in Claims 1-11 of the '618 Patent, thus rendering them unpatentable.

67. Based on my review of the above cited prior art references, Claims 1-11 are unpatentable as indicated below in Table 1:

68. Table 1 – Grounds of Unpatentability

Ground	'618 Patent	Basis for Invalidity
Ground 1A (G1)	1-3	Obvious under §103(a) over Van Antwerp
Ground 1B (G2)	4, 5	Obvious under §103(a) over Van Antwerp in view of Bostek
Ground 1C (G3)	6-9	Obvious under §103(a) over Van Antwerp in view of Moon
Ground 1D (G4)	10, 11	Obvious under §103(a) over Van Antwerp in view of Hamade
Ground 2A (G5)	1-8, 10-11	Obvious under §103(a) over Schneider

Ground 2B (G6)	9	Obvious under §103(a) over Schneider in view of McDaniel
Ground 3A (G7)	1-9	Obvious under §103(a) over Drevon
Ground 3B (G8)	10, 11	Obvious under §103(a) over Drevon in view of Schneider

X. CLAIM CONSTRUCTION

69. In conducting my analysis of the asserted claims of the '618 Patent, I have applied the legal understandings I set out below regarding claim constructions consistent with the "broadest reasonable construction" standard described above, and offer them only for this *Inter Partes* Review. The claim constructions do not necessarily reflect the appropriate claim constructions to be used in litigation proceedings, such as litigation in a district court, where a different standard applies.

70. My understanding is that the broadest definition for the term ("facilitating the removal of a fingerprint by vaporization") that is supported by the specification of the '618 Patent is "enabling a bioorganic material deposited by an organism through touching a lipase associated substrate or coating to transition from an initial quantity of visually apparent bioorganic material being on such substrate or coating to a lesser quantity of visually apparent bioorganic material being thereon."

71. I understand that, under the BRI claim construction, a claim in an unexpired patent shall be given its broadest reasonable construction in light of the specification of the patent; claim terms are given their ordinary and customary meaning as would be understood by one of ordinary skill in the art in the context of the entire disclosure; an inventor may rebut that presumption by providing a definition of the term in the specification with reasonable clarity, deliberateness, and precision; and, in the absence of such a definition, limitations are not to be read from the specification into the claims.

72. Further, it is my understanding that the basis in the '618 Patent for the BRI claim construction includes the claims, the disclosure (e.g., claims scope supported by the disclosure, Applicant serving as its own lexicographer, and inherent functionality of disclosed compositions), as well as statements made in the prosecution history of the '063 Application

1. CLAIM TERM LEXICOGRAPHER

73. I understand the following with respect to '063 Application Applicant choosing to be its own lexicographer with respect to the term "fingerprint" and, where applicable, have provided my opinion regarding same.

74. It is my understanding as provided for by MPEP 2111.01, "an applicant is entitled to be his or her own lexicographer and may rebut the presumption that claim terms are to be given their ordinary and customary meaning by

clearly setting forth a definition of the term that is different from its ordinary and customary meaning(s) in the specification at the time of filing." See *In re Paulsen,* 30 F.3d 1475, 1480, 31 USPQ2d 1671, 1674 (Fed. Cir. 1994) (holding that an inventor may define specific terms used to describe invention, but must do so "with reasonable clarity, deliberateness, and precision" and, if done, must " 'set out his uncommon definition in some manner within the patent disclosure' so as to give one of ordinary skill in the art notice of the change" in meaning) (quoting *Intellicall, Inc. v. Phonometrics, Inc.,* 952 F.2d 1384, 1387-88, 21 USPQ2d 1383, 1386 (Fed. Cir. 1992)).

75. The'063 Application Applicant has served as its own lexicographer in defining the term "fingerprint" as recited in independent claim 1. Specifically, the '618 Specification discloses the following:

> A fingerprint as defined herein is a bioorganic stain, mark, or residue left behind after an organism touches a substrate or coating. A fingerprint is not limited to marks or residue left behind after a substrate is touched by a finger. Other sources of bioorganic stains are illustratively, palms, toes, feet, face, any other skin surface area, hair, stains from fats used in cooking such as cis-fatty acids, or fatty acids from any other source. ('618 Patent at 3:1-9)

76. In being its own lexicographer,'063 Application Applicant has defined what a "fingerprint" is in very broad terms. A fingerprint has been defined to not be only marks or residue left behind after a substrate is touched by a finger. Conversely, a fingerprint has been defined to be a bioorganic stain, mark, or residue left behind after an organism touches a substrate or coating, wherein sources of such bioorganic stains are illustratively, palms, toes, feet, face, any other skin surface area, hair, stains from fats used in cooking such as cis-fatty acids, or fatty acids from any other source. Moreover, the disclosure of "fatty acids from any other source" in conjunction with the invention of the '618 Patent not being restricted to any particular intended applications or products (see section IV.B) clearly support that the term "fingerprint" has been given a substantially broader and different meaning than the plain and ordinary meaning of a mark left behind after a surface is touched with a finger.

2. CLAIM SCOPE SUPPORTED BY THE DISCLOSURE

77. I understand the following with respect to claim scope supported by the disclosure of the '618 Patent and, where applicable, have provided my opinion regarding same.

78. A first consideration regarding claim scope supported by the disclosure is the test procedure for verifying removal of a fingerprint. The disclosures of the

'618 Patent are limited to use of visual verification for making a determination that a fingerprint has been removed from a substrate or coating having a lipase associated therewith in accordance with the disclosures of the '618 Patent. For example, the '618 Patent discloses, "The surface temperature is optionally raised to such a level that the breakdown products volatilize to the point of no visual material remaining on the substrate within 24 hours. Optionally, the temperature is raised to such a level that the breakdown products are removed to the point of no visual material remaining on the substrate within 0.5 to 3 hours, inclusive" (*Id.* at 10:56-62) and "Heat is optionally applied until the breakdown products volatilize to the point of no visual material remaining on the substrate" (*Id.* at 10:66-11:1). In this regard, the '618 Patent presents no disclosure other than visual observation for scientifically verifying that any component of a fingerprint has been enzymatically degraded by a lipase associated with a substrate or coating and subsequently has been removed by vaporization from such lipase associated substrate or coating.

79. As supported by the disclosures in Buchannan [Ex. 1013], it is my opinion that it is well known that fingerprints on a substrate or coating become less visually apparent over time regardless of whether or not the substrate or coating has a lipase associated therewith. Buchanan also describes that the

mechanism by which fingerprints disappear over time is by vaporization, and that fingerprints can disappear by vaporization either at ambient temperature or with mild heating. Buchanan further demonstrates that fingerprints containing a larger fraction of more volatile components, such as lower molecular weight fatty acids, alcohols, and esters (exemplified by the fingerprints of children), will vaporize more quickly than fingerprints containing less volatile components (exemplified by the fingerprints of adults). (Buchanan at Abstract:1-8, 89:21-29; 91:1-7) (See *Id.* at ¶¶ 33-34)

80. A second consideration regarding claim scope supported by the disclosure is incubation temperature. The '618 Patent presents information relating to the vaporization of breakdown products at stated temperatures and ranges of temperatures. (*Id.* at 10:43-11:3). However, the only temperatures at which lipase associated substrates/coatings in accordance with the disclosures of the '618 Patent were evaluated in working examples are room temperature ("RT") and 65° C. (*Id.* at 12:12-21 and 12:29-33).

81. Notably, room temperature is not quantitatively defined in the '618 Patent.

82. Although it is disclosed in the '618 Patent that the claimed "fingerprint removal" from a lipase associated substrate or coating can take place at "any temperature whereby the lipase is active," "4°C", "25°C", "ambient temperature", and "between 40°C and 120°C" (*Id.* at 2:43-54; 10:43-49),

Petitioner suggests that the working examples do not support such an assertion. For example, with respect to working Example 2 and referring to FIG. 1, the '618 Patent discloses, "Fingerprinted panels are incubated at room temperature for at least 24 hours. A control panel is coated with the coating of Example 1 that is free of enzyme. After this first incubation period, the coated substrate is incubated in an oven at a temperature of 65° C or higher for 1 to 6 hours. FIG.1 demonstrates that incubation of the enzyme coated panels at 65° C for two hours facilitates complete removal of fingerprints. (B: control; L: lipase; LA: combined lipase and amylase in coating.)" (Id. at 12:12-21).

83. It is my opinion that the test results shown in FIG. 1 of the '618 Patent provide no evidence that any amount of fingerprint removal occurred at room temperature because there is no test sample from before and after being incubated at room temperature for at least 24 hours.

84. It is my opinion that, in FIG. 2, there is no evidence that any amount of fingerprint removal occurred at room temperature because there is no test sample from before and after being incubated at room temperature for 3 days.

85. It is my opinion that FIG. 2 of the '618 Patent shows evidence that fingerprint removal only occurred after the room temperature incubated

test samples were subsequently incubated at 65° C. Specifically, as shown in FIG. 2, there is no visual indication of evidence at 3 days of initial incubation (i.e., reaction period) and at 0 days of incubation at 65° C that any amount of fingerprint removal has occurred at room temperature. It is only after the test samples are incubated at 65° C for 2.5 hours that there is less visual material remaining on the test panels than at earlier periods of time. Thus, Petitioner submits that the working examples only provide evidence of fingerprint removal from a lipase associated substrate or coating after being exposed to heat at 65° for at least 2.5 hours.

86. It is my opinion that, because the term "breakdown products" is not explicitly defined in the written description, breakdown products is a term that is analogous to the recited term "degradation products" (*Id.* at 2:51), which refers to the resulting products from lipase catalyzing the hydrolysis, esterification, or transesterification of lipids (*Id.* at 2:43-47).

87. The term ambient temperature is also referred to in the disclosure describing one of the working examples. (*Id.* at 12:30). However, in FIG. 2, which shows results of one of the working examples, it is indicated that an associated portion of the experimentation of such working example was carried out at room temperature (RT).

88. Thus, it is my opinion that the disclosures of the '618 Patent imply that ambient temperature and room temperature (RT) are the same.

XI. UNPATENTABILITY OF THE '618 PATENT CLAIMS

A. GROUND 1A: CLAIMS 1-3 ARE UNPATENTABLE UNDER 35 U.S.C. § 103(A) AS OBVIOUS OVER VAN ANTWERP

89. It is my opinion that the cited sections in **Table 2** below, which are also included in the IPR Petition, establish that the disclosures of Van Antwerp in light of inherent functionality of a lipase on a component of a fingerprint renders Claims 1-3 obvious.

1. Independent Claim 1

90. Van Antwerp describes a catheter made from a polymeric material (*Id.* at 3:41-44; 3:65-67), which is substrate upon which a coating can be provided.

91. Van Antwerp discloses providing a lipolytic enzyme coating on an article for the purpose of enzymatically dissolving a lipid-based substance that may come into contact with such article. (Van Antwerp [Ex. 1006] at 4:8-21; FIG. 4:enzyme 21 on exterior surface). Van Antwerp describes providing a stable and substantially immobilized enzyme coating on interior and exterior surfaces of the catheter (*Id.* at 2:34-42; FIG. 4:enzyme 21 on exterior surface), that the enzyme can be a lipolytic enzyme (*Id.* at 2:38-42) and that the lipolytic enzyme can be phospholipase (*Id.* at 4:18-

20).Van Antwerp also teaches that the lipolytic enzyme is capable of dissolving a lipid-based substance, (*Id.* at 4:8-21; 6:14-18; FIG. 4:enzyme 21 on exterior surface), such dissolving being a form of degradation caused by enzymatic activity. (*Id.* at 4:18-26).

92. Van Antwerp does not explicitly disclose facilitating the removal of a fingerprint by vaporization from a lipase-associated substrate or coating when contacted by a fingerprint.

93. Although Van Antwerp does not explicitly disclose facilitating the removal of a fingerprint by vaporization from the lipase associated substrate or coating when contacted by a fingerprint, Van Antwerp discloses that the purpose of the lipolytic enzyme coating on the polymeric catheter is dissolving a lipid-based substance, (*Id.* at 4:8-21; 6:14-18; FIG. 4:enzyme 21 on exterior surface), such dissolving being a form of enzymatic degradation. (*Id.* at 4:18-26).

94. In my opinion it would have been obvious to a POSITA at the time of the invention of the '618 Patent to facilitate the removal of a fingerprint by vaporization using a lipase entrapped in or adhered to a substrate or coating based on the disclosures of Van Antwerp.

95. It is my opinion that the lipid degradation functionality is inherently present in a lipase associated with a substrate or coating (e.g., the phospholipase

coated polymeric catheter of Van Antwerp), and that a POSITA would understand that this lipid degradation activity in the lipase-associated coating would facilitate removal of a fingerprint because one or more components of the fingerprint is a lipid-based substance well-known to be enzymatically degraded by a lipase.

96. Therefore, the disclosures of Van Antwerp, in light of inherent functionality of a lipase enzyme on components of a "fingerprint" based on the inherent activity of a lipase to degrade lipid-based substances such as those known to be components of fingerprints (See Section VI(B) ¶ 35 above), render obvious the invention as recited in Claim 1, as indicated through the respective citations in Table 2 below.

2. Dependent Claim 2

97. Van Antwerp discloses that enzymes can be coated onto the catheter in a well-known manner (e.g., "The capsules 26 are then bonded to the polymeric catheter material by silicone chemistry") whereby such enzyme is covalently attached to the catheter. (*Id.* at 5:29-43; 5:59-6:9).

98. Thus, it is my opinion that Van Antwerp teaches the limitations of dependent Claim 2, as indicated through the respective citations in Table 2 below.

3. Dependent Claim 3

99. Van Antwerp discloses that enzyme can be coated onto the catheter in a well-known manner (e.g., "immersion of catheter 10 into a prepared enzyme slurry or emulsion 20. In this regard, the enzyme is commonly available in particulate form, having a particle size ranging on the order of one to fifteen microns. The enzyme particles are mixed in a liquid carrier such as water to produce the emulsion 20 shown in FIG. 3. Upon withdrawal of the catheter 10 from the enzyme emulsion 20, the catheter surface is allowed to dry resulting in adherence of the enzyme to the catheter in a micellar array of microsphere particles 21") whereby such enzyme is non-covalently adhered to the catheter. (*Id.* at 4:36-47; 2:46-50).

100. It is my opinion that Van Antwerp teach the limitation of dependent Claim 3, as indicated through the respective citations in Table 2 below.

101. It is therefore my opinion that the disclosures of Van Antwerp in light of inherent functionality of a lipase enzyme on components of a "fingerprint" render Claims 1-3 obvious and unpatentable.

102. **Table 2 - Claims 1-3 Mapping Based on Van Antwerp**

1. [P1] A method of facilitating the removal of a fingerprint on a substrate or a coating comprising:	Van Antwerp at 4:8-21; FIG. 4:enzyme 21 on exterior surface See also sections IV. D and IV.E of the Petition.
Element [A1] providing a substrate or a coating;	Van Antwerp at 3:41-44; 3:65-67

Element [B1] associating a lipase with said substrate or said coating such that said lipase is capable of enzymatically degrading a component of a fingerprint,	Van Antwerp at 2:34-42; FIG. 4:enzyme 21 on exterior surface; 4:8-26; 6:14-18 See also sections IV. D and IV.E of the Petition.
Element [C1] facilitating the removal of a fingerprint by vaporization from the lipase associated substrate or coating when contacted by a fingerprint.	Van Antwerp at 4:8-26; 6:14-18; FIG. 4:enzyme 21 on exterior surface See also sections IV. E, IV.F and IV.D of the Petition.
2. The method of claim 1 wherein said lipase is covalently attached to said substrate or to said coating.	Van Antwerp at 5:29-43; 5:59-6:9
3. The method of claim 1 wherein said lipase is non-covalently adhered to or admixed into said substrate or said coating.	Van Antwerp at 4:36-47; 2:46-50; FIG. 3

B. GROUND 1B: CLAIMS 4 AND 5 ARE UNPATENTABLE UNDER 35 U.S.C. § 103(A) AS OBVIOUS OVER VAN ANTWERP IN VIEW OF BOSTEK

103. It is my opinion that the cited sections in **Table 3** below, which are also included in the IPR Petition, establish that Van Antwerp as applied above to Claim 1 in view of Bostek teaches all of the limitations in Claims 4 and 5 and render these claims obvious and unpatentable.

104. It is my opinion that a POSITA would have been motivated, or would have found it obvious, at the time that the invention was made to combine the disclosures of Van Antwerp and Bostek because the disclosures of Van Antwerp and Bostek are in overlapping technical fields, address similar

technical disclosure relating to utilization and/or construction of catheters, and present motivating and/or suggesting disclosure for such combination.

1. Dependent Claims 4 and 5

105. Van Antwerp discloses that a catheter is used for delivering medical fluids to or drawing body fluids from a patient that contains a lipase in a coating for the purpose of degrading lipid-based substances. (*Id.* at 1:21-23; 8-21; FIG. 4:enzyme 21 on exterior surface). Van Antwerp does not explicitly disclose heating said substrate or said coating or applying heat to a surface of said substrate or said coating subsequent to being contacted by a fingerprint.

106. Bostek [Ex. 1009] discloses infusing intravenous (IV) fluid that is heated above room temperature into a patient through a catheter and that such infusing is performed for a period of at least 2 hours (*Id.* at pg. 564, col. 1, ln. 13-16; pg. 564, col. 2, ln. 1-10; pg. 564, col. 2, ln. 14-pg. 565, ln. 12), which would result in heating of the catheter (i.e., Bostek's catheter heating functionality).

107. It is my opinion that, in view of these disclosures of Van Antwerp and Bostek, a POSITA would have found it obvious at the time of the invention of the '618 Patent was made to combine the disclosures of Van Antwerp with Bostek's catheter heating functionality.

108. It is my opinion that a motivation for a POSITA to make such a combination is that Van Antwerp teaches the underlying use of using catheters to deliver medical fluids into a patient and Bostek provides specific examples of implementing such use of a catheter.

109. It is my opinion that Van Antwerp as applied above to Claim 1 in view of Bostek teaches the limitation of dependent Claims 4 and 5, as indicated through the respective citations in Table 3 below.

110. It is therefore my opinion that the combination of Van Antwerp as applied above to Claim 1 in view of Bostek renders claims 4 and 5 obvious and unpatentable.

111. **Table 3 – Claims 4 and 5 Mapping Based on Van Antwerp in view of Bostek**

4. The method of claim 1 comprising heating said substrate or said coating or applying heat to a surface of said substrate or said coating subsequent to being contacted by a fingerprint.	Van Antwerp at 1:21-23 Bostek at pg. 564, col. 1, ln. 13-16; pg. 564, col. 2, ln. 1-10; pg. 564, col. 2, ln. 14-pg. 565, ln. 12
5. The method of claim 4 wherein said heating is for at least 30 minutes.	Van Antwerp at 1:21-23; 1:37-40 Bostek at pg. 564, col. 1, ln. 13-16; pg. 564, col. 2, ln. 1-10; pg. 564, col. 2, ln. 14-pg. 565, ln. 12

C. GROUND 1C: CLAIM 6-9 ARE UNPATENTABLE UNDER 35 U.S.C. § 103(A) AS OBVIOUS OVER VAN ANTWERP IN VIEW OF MOON

112. It is my opinion that the cited sections in **Table 4** below, which are also included in the IPR Petition, establish that Van Antwerp as applied above to Claim 1 in view of Moon teaches all of the limitations in Claims 6-9 and render these claims obvious and unpatentable.

113. It is my opinion that a POSITA would have been motivated, or would have found it obvious, at the time that the invention was made to combine the disclosures of Van Antwerp and Moon because the disclosures of Van Antwerp and Moon are in overlapping technical fields, address similar technical disclosure relating to utilization and/or construction of catheters, and present motivating and/or suggesting disclosure for such combination.

1. Dependent Claims 6 and 8

114. Van Antwerp discloses that the catheter can be made from a polymeric material and that polyethylene is an example of such a polymeric material. (*Id.* at 3:65-67).

115. It is my opinion that polyethylene is well known to be an organic crosslinkable polymer resin.

2. Dependent Claim 7

116. Although Van Antwerp discloses that the catheter can be made from an organic crosslinkable polymer resin (e.g., polyethylene), Van Antwerp does not explicitly disclose that the organic crosslinkable polymer resin

comprises a functional group of acetoacetate, acid, amine, carboxyl, epoxy, hydroxyl, isocyanate, silane, vinyl, or combinations thereof.

117. Moon discloses polymeric resin compositions suitable for use in medical supplies such as a catheter that provide improved antimicrobial characteristics (Moon [Ex. 1006] at 0059: 1-2, 0059:12-16; 0115:1-8), and that polymeric resins that may be used in the composition can include a functional group of isocyanate, hydroxyl, or epoxy (*Id.* at 0119:1-12, 112:1-25).

118. It is my opinion that, in view of these disclosures of Moon, a POSITA would have found it obvious at the time of the invention of the '618 Patent was made to modify the polymeric material of Van Antwerp to include at least one of the isocyanate, hydroxyl, or epoxy functional groups of Moon.

119. It is my opinion that one motivation for such modification is that Moon discloses that the polymer resins thereof can be used for making medical supplies such as a catheter (*Id.* at 0059: 1-2, 0059:12-16) and that another motivation for such modification is seeking a polymeric material that exhibits improved antimicrobial characteristics.

3. Dependent Claim 9

120. Although Van Antwerp discloses that the catheter can be made from a polymeric material (*Id.* at 3:65-67), which can be an organic crosslinkable polymer (e.g., polyethylene), Van Antwerp does not explicitly disclose that such polymeric material is a hydroxyl-functionalized acrylate resin.

121. Moon discloses polymeric resin compositions suitable for use in medical supplies such as a catheter that provide improved antimicrobial characteristics (Moon [Ex. 1006] at 0059:1-2, 0059:12-16; 0115:1-8) and that such antimicrobial polymeric resin compositions can be a hydroxyl-functionalized acrylate resin (*Id.* at 0112:1-25; 0115:1-8; 0120:1-0122:6).

122. It is my opinion that, in view of these disclosures of Moon, a POSITA would have found it obvious at the time of the invention of the '618 Patent was made to modify the polymeric material of Van Antwerp to be a hydroxyl-functionalized acrylate resin of Moon.

123. It is my opinion that one motivation for such modification is that Moon discloses that the polymer resins thereof can be used for making medical supplies such as a catheter (*Id.* at 0059: 1-2, 0059:12-16) and that another motivation for such modification is seeking a polymeric material that exhibits improved antimicrobial characteristics.

124. It is therefore my opinion that the combination of Van Antwerp as applied above to Claim 1 in view of Moon renders claims 6-9 obvious and unpatentable.

125. **Table 4 – Claims 6-9 Mapping Based on Van Antwerp in view of Moon**

6. The method of claim 1 wherein said substrate or said coating comprises an organic crosslinkable polymer resin.	Van Antwerp at 3:65-67
7. The method of claim 6 wherein said organic crosslinkable polymer resin comprises a functional group of acetoacetate, acid, amine, carboxyl, epoxy, hydroxyl, isocyanate, silane, vinyl, or combinations thereof.	Van Antwerp at 3:65-67 Moon at 0059: 1-2, 0059:12-16; 0115:1-8, 0119:1-12, 112:1-25
8. The method of claim 6 wherein said organic crosslinkable polymer resin is aminoplasts, melamine formaldehydes, carbamates, polyurethanes, polyacrylates, epoxies, polycarbonates, alkyds, vinyls, polyamides, polyolefins, phenolic resins, polyesters, polysiloxanes, or combinations thereof.	Van Antwerp at 3:65-67
9. The method of claim 6 wherein said organic crosslinkable polymer is a hydroxyl-functionalized acrylate resin.	Van Antwerp at 3:65-67 Moon at 0059:1-2, 0059:12-16; 0115:1-8, 0112:1-25; 0120:1-0122:6

D. GROUND 1D: CLAIMS 10 AND 11 ARE UNPATENTABLE UNDER 35 U.S.C. § 103(A) AS OBVIOUS OVER VAN ANTWERP IN VIEW OF HAMADE

126. It is my opinion that the cited sections in **Table 5** below, which are also included in the IPR Petition, establish that Van Antwerp as applied above to

Claim 1 in view of Hamade teaches all of the limitations in Claims 10 and 11 and render these claims obvious and unpatentable.

127. It is my opinion that a POSITA would have been motivated, or would have found it obvious, at the time that the invention was made to combine the disclosures of Van Antwerp and Hamade because the disclosures of Van Antwerp and Hamade are in overlapping technical fields, address similar technical disclosure relating to utilization and/or construction of articles subjected to microbial contamination during use in hospital settings, and present motivating and/or suggesting disclosure for such combination.

1. Dependent Claims 10 and 11

128. Van Antwerp discloses providing a stable and substantially immobilized enzyme coating on interior and exterior surfaces of the catheter (*Id.* at 2:34-42; FIG. 4: enzyme 21 on exterior surface), that the enzyme can be a lipolytic enzyme (*Id.* at 2:38-42) and that the lipolytic enzyme can be phospholipase (*Id.* At 4:18-20).

129. Although Van Antwerp discloses that the enzyme can be phospholipase, Van Antwerp does not explicitly disclose that the enzyme is lipoprotein lipase, acylglycerol lipase, hormone-sensitive lipase, phospholipase Al, phospholipase A2, phospholipase C, phospholipase D, phosphoinositide phospholipase C, a lysophospholipase, or a galactolipase.

130. Hamade discloses a coating composition comprising a film-forming resin, an enzyme, and a substrate, said enzyme being capable of reacting with said substrate to produce a compound having antimicrobial activity. (Hamade [Ex. 1007] at 7:31-35) and that the enzyme can be a lipase such as triacylglycerol lipase or lipoprotein lipase. (*Id.* at 4:7-15).

131. It is my opinion that it would have been obvious to a POSITA at the time that the invention of the '618 Patent was made to modify the enzyme of the enzyme-coated catheter disclosed by Van Antwerp to be lipoprotein lipase or triacylglycerol lipase disclosed by Hamade.

132. It is my opinion that a motivation for such modification is the POSITA seeking enzymes the exhibit enzymatic activity against various lipids and both lipoprotein lipase and triacylglycerol lipase being well known to enzymatically degrade components of bioorganic stains such as, for example lipids, fats, cellular debris and the like (e.g., phospholipase disclosed by Van Antwerp is well known to have comparable performance as lipoprotein lipase and triacylglycerol lipase with respect to enzymatically degrading lipid-based substances.)

133. It is therefore my opinion that the combination of Van Antwerp as applied above to Claim 1 in view of Hamade renders claims 10 and 11 obvious and unpatentable.

134. Table 5 – Claims 10 and 11 Mapping Based on Van Antwerp in view of Hamade

10. The method of claim 1 wherein said lipase is lipoprotein lipase, acylglycerol lipase, hormone-sensitive lipase, phospholipase A1, phospholipase A2, phospholipase C, phospholipase D, phosphoinositide phospholipase C, a lysophospholipase, or a galactolipase.	Van Antwerp at 2:34-42; FIG. 4: enzyme 21 on exterior surface; 4:18-20 Hamade at 7:31-35; 4:7-15
11. The method of claim 1 wherein said lipase is a triacylglycerol lipase.	Van Antwerp at 2:34-42; FIG. 4: enzyme 21 on exterior surface; 4:18-20 Hamade at 7:31-35; 4:7-15

E. GROUND 2A: CLAIMS 1-8 AND 10-11 ARE UNPATENTABLE UNDER 35 U.S.C. § 103(A) AS OBVIOUS OVER SCHNEIDER

135. It is my opinion that the cited sections in **Table 6** below, which are also included in the IPR Petition, establish that the disclosures of Schneider in light of inherent functionality of a lipase on a component of a fingerprint renders Claims 1-8 and 10-11 obvious.

1. Independent Claim 1

136. Schneider discloses "methods for treating a surface contacted by fouling organisms or a surface at risk of such contact, said method comprising the steps of contacting the surface with a composition according to the invention with an effective amount of said composition or coating

composition, wherein said contacting results in eliminating said fouling or at least reducing said fouling." (Schneider [Ex. 1004] at 0266:1-7).

137. Schneider discloses fouling to be "microbial growth and/or the formation of a bio-film on objects coated with the composition." (*Id.* at 0125:3-4).

138. Schneider discloses that cell wall lipids and other lipid associated macromolecules are components of microbial organisms (*Id.* at 0072:1-5).

139. Schneider discloses coatings to be applied to a surface of an article for providing antifouling (e.g., antimicrobial) activity. (*Id.* at 0050:1-8; 0125:1-5; 0247:1-4; 0248:1-3, 253:1-13; 0262:1-4, 0269:1-4).

140. Schneider discloses that the coating compositions include at least one enzyme and that the at least one enzyme can be a lipase. (*Id.* at 0050:1-4; 0052:1-8; 0074:1-3; 0088:1-0090:3; 0096:1-3) and that lipases are capable of degrading cell wall lipids and other and other lipid associated macromolecules at the surface of microbial organisms. (*Id.* at 0072:1-5).

141. Although Schneider does not explicitly disclose facilitating the removal of a fingerprint by vaporization from the lipase associated substrate or coating when contacted by a fingerprint, Schneider discloses that lipases are capable of degrading cell wall lipids and other lipid associated macromolecules at the surface of microbial organisms. (*Id.* at 0072:1-5).

142. It is my opinion that, at the time the invention of the '618 Patent was made, a POSITA would have appreciated that the enzyme-associated coating as disclosed by Schneider is capable of facilitating the removal of a bioorganic stain (i.e., a fingerprint) by vaporization when such enzyme is the disclosed lipase and such lipase coating is present in an environment that supports vaporization of the enzymatically degraded component(s) of the bioorganic stain.

143. It is my opinion that fingerprint removal functionality in accordance with the claimed invention of the '618 Patent is passive and is inherently present in a lipase associated substrate or coating (e.g., the enzyme-associated coating of Schneider) because one or more components of the bioorganic stain is well-known to be enzymatically degraded by a lipase in a manner that allows for its vaporization when in an environment that would support such vaporization.

144. Thus, it is my opinion that the Schneider in light of inherent functionality of a lipase enzyme on components of a "fingerprint" renders obvious the invention as recited in Claim 1, as indicated through the respective citations in Table 6 below.

2. Dependent Claim 2

145. Schneider discloses immobilization of enzymes within the coating (*Id.* at 0110:1-12, 0247:1-4) and, as discussed in reference to Claims 1, that the

enzyme can be a lipase. For example, Schneider explicitly discloses that immobilization includes enzymes immobilized on polymer matrices, among other forms. (*Id.* at 0110:8-12)

146. It is my opinion that, in view of the disclosures of Schneider with respect to immobilization of enzymes, a POSITA would understand that the lipase enzyme of the coating of Schneider can be covalently attached to one or more elements of such a coating (e.g., a binder thereof) and would understand that the lipase enzyme would be covalently attached to the coating of Schneider.

3. Dependent Claim 3

147. Schneider discloses that the at least one enzyme of the disclosed coatings can be admixed into such coating (*Id.* at 0263:1-4; 0110:1-12).

4. Dependent Claims 4 and 5

148. Schneider discloses use of the enzyme containing coating in applications such as outdoor wood work and external surface of a central heating system (*Id.* at 0249:1-3) and a pipe for ventilation (*Id.* at 0269:1-4).

149. It is my opinion that it would have been well-known to a POSITA at the time the invention of the '618 Patent was made that articles in such applications (e.g., outdoor wood work and external surface of a central heating system) can be subjected to bioorganic stains and reside in and/or

operate under conditions in which surfaces thereof upon which the coating of Schneider can be applied become heated. For example, a wood table exposed to sunlight and/or ambient air can become heated for several hours each day, surfaces of a central heating system can be exposed to heated air for several hours each day, and a ventilation pipe for exhausting cooking fumes can be exposed to heated (and cooking oil/fatty acid laden) air for several hours each day.

5. Dependent Claim 6

150. Schneider discloses that compositions and/or paints thereof (i.e., enzyme-containing polymeric coatings) may be polymeric, oligomeric, monomeric, and may contain cross-linkers or cure promoters as needed. (*Id.* at 0225:1-3) and that enzyme-containing polymeric coatings thereof can comprise one or more of drying oils, alkyd resins, epoxy resins, urethane resins, polyester resins, vinyl resins, and phenolic resins (*Id.* at 0253:1-13).

151. It is my opinion that drying oils, alkyd resins, epoxy resins, urethane resins, polyester resins, vinyl resins, and phenolic resins are well known to be an organic crosslinkable polymer resin.

6. Dependent Claim 7

152. Schneider discloses that the enzyme-containing polymeric coatings thereof can comprise epoxy resins, urethane resins, polyester resins, vinyl resins,

drying oils, alkyd resins, and phenolic resins, derivatives and mixtures thereof (Id. at 0253:1-13).

153. It is my opinion that, in view of these disclosures of Schneider, a POSITA would have found it obvious at the time of the invention of the '618 Patent was made that such enzyme-containing polymeric coatings can be an organic crosslinkable material that comprises a functional group including at least one of acid, amine, carboxyl, epoxy, hydroxyl, and isocyanate, given that epoxy resins, urethane, and phenolic resins typically comprise epoxy, isocyanate, and hydroxyl functional groups, respectively.

7. Dependent Claim 8

154. Schneider discloses that the enzyme-containing polymeric coatings thereof can comprise one or more of epoxy resins, urethane resins, polyester resins, vinyl resins, and phenolic resins (*Id.* at 0253:1-13).

8. Dependent Claims 10 and 11

155. Schneider discloses that the lipase of the enzyme-containing polymeric coatings thereof can be lipoprotein lipase. (*Id.* at 0074:1-3) and that the lipase of the enzyme-containing polymeric coatings thereof can be triacylglycerol lipase. (*Id.* at 0074:1-3).

156. It is therefore my opinion that the Schneider in light of inherent functionality of a lipase enzyme on components of a "fingerprint" renders Claims 1-8, 10 and 11 obvious and unpatentable.

157. Table 6 - Claims 1-8 and 10-11 Mapping Based on Schneider

1. [P1] A method of facilitating the removal of a fingerprint on a substrate or a coating comprising:	Schneider at 0266:1-7; 0125:3-4; 0072:1-5 See also sections IV. D and IV.E of the Petition.
Element [A1] providing a substrate or a coating;	Schneider at 0050:1-8; 0125:1-5; 0247:1-4; 0248:1-3, 253:1-13; 0262:1-4, 0269:1-4
Element [B1] associating a lipase with said substrate or said coating such that said lipase is capable of enzymatically degrading a component of a fingerprint,	Schneider at 0050:1-4; 0052:1-8; 0074:1-3; 0088:1-0090:3; 0096:1-3; 0072:1-5
Element [C1] facilitating the removal of a fingerprint by vaporization from the lipase associated substrate or coating when contacted by a fingerprint.	Schneider at 0072:1-5 See also sections IV. E, IV.F and IV.D of the Petition.
2. The method of claim 1 wherein said lipase is covalently attached to said substrate or to said coating.	Schneider at 0110:1-12, 0247:1-4
3. The method of claim 1 wherein said lipase is non-covalently adhered to or admixed into said substrate or said coating.	Schneider at 0263:1-4; 0110:1-12
4. The method of claim 1 comprising heating said substrate or said coating or applying heat to a surface of said substrate or said coating subsequent to being contacted by a fingerprint.	Schneider at 0249:1-3; 0269:1-4

5. The method of claim 4 wherein said heating is for at least 30 minutes.	Schneider at 0249:1-3; 0269:1-4
6. The method of claim 1 wherein said substrate or said coating comprises an organic crosslinkable polymer resin.	Schneider at 0225:1-3; 0253:1-13
7. The method of claim 6 wherein said organic crosslinkable polymer resin comprises a functional group of acetoacetate, acid, amine, carboxyl, epoxy, hydroxyl, isocyanate, silane, vinyl, or combinations thereof.	Schneider at 0253:1-13
8. The method of claim 6 wherein said organic crosslinkable polymer resin is aminoplasts, melamine formaldehydes, carbamates, polyurethanes, polyacrylates, epoxies, polycarbonates, alkyds, vinyls, polyamides, polyolefins, phenolic resins, polyesters, polysiloxanes, or combinations thereof.	Schneider at 0253:1-13
10. The method of claim 1 wherein said lipase is lipoprotein lipase, acylglycerol lipase, hormone-sensitive lipase, phospholipase A1, phospholipase A2, phospholipase C, phospholipase D, phosphoinositide phospholipase C, a lysophospholipase, or a galactolipase.	Schneider at 0074:1-3
11. The method of claim 1 wherein said lipase is a triacylglycerol lipase.	Schneider at 0074:1-3

F. GROUND 2B: CLAIM 9 IS UNPATENTABLE UNDER 35 U.S.C. § 103(A) AS OBVIOUS OVER SCHNEIDER IN VIEW OF MCDANIEL

158. It is my opinion that the cited sections in **Table 7** below, which are also included in the IPR Petition, establish that Schneider as applied above to

Claim 1 in view of McDaniel teaches all of the limitations in Claim 9 and render this claim obvious and unpatentable.

159. It is my opinion that a POSITA would have been motivated, or would have found it obvious, at the time that the invention was made to combine the disclosures of Schneider and McDaniel because the disclosures of Schneider and McDaniel are in overlapping technical fields, address similar technical disclosure relating to utilization and/or construction of articles subjected to microbial contamination during use in hospital settings, and present motivating and/or suggesting disclosure for such combination.

1. Dependent Claim 9

160. Although Schneider discloses that compositions and/or paints thereof (i.e., enzyme-containing polymeric coatings) may be polymeric, oligomeric, monomeric, and may contain cross-linkers or cure promoters as needed (*Id.* at 0225:1-3) and that the enzyme-containing polymeric coatings thereof may have any suitable surface coating material incorporated therein and can comprise acrylic resins and methacrylate resins, epoxy resins, urethane resins, polyester resins, vinyl resins, and phenolic resins. and derivatives and mixtures thereof (*Id.* at 0253:1-13), Schneider does not explicitly disclose that the enzyme-containing polymeric coatings thereof

include an organic crosslinkable polymer that is a hydroxyl-functionalized acrylate resin.

161. McDaniel discloses enzyme-containing polymeric coatings (McDaniel [Ex. 1004] at 0023:1-6; 0046:1-9; 0379:1-4, 0094:1-4) and that such enzyme-containing polymeric coatings include an organic crosslinkable polymer that is a hydroxyl-functionalized acrylate resin (*Id.* at 0379:1-4; 0503:1-16; -0504:1-20; 0454:1-6; 0510:2-18; 0512:2-8).

162. It is my opinion that a POSITA would have found it obvious at the time the invention of the '618 Patent was made to combine the enzyme-containing polymeric coatings of Schneider with the hydroxyl-functionalized acrylate resin of McDaniel.

163. It is my opinion that a motivation for such combination is that Schneider provides the suggestion for such combination through its disclosure of other functional groups for polymeric resins and associated benefits thereof and the POSITA would seek material compositions that are well-known to provide desirable performance for enzyme-containing polymeric coatings and a hydroxyl-functionalized acrylate resin is well-known to provide desirable performance for enzyme-containing polymeric coatings.

164. It is therefore my opinion that the combination of Schneider as applied above to Claim 1 in view of McDaniel renders claim 9 obvious and unpatentable.

165. Table 7 - Claim 9 Mapping Based on Schneider in view of McDaniel

9. The method of claim 6 wherein said organic crosslinkable polymer is a hydroxyl-functionalized acrylate resin.	Schneider at 0225:1-3; 0253:1-13 McDaniel at 0023:1-6; 0046:1-9; 0379:1-4, 0094:1-4; 0503:1-16; 0504:1-20; 0454:1-6; 0510:2-18; 0512:2-8

G. GROUND 3A: CLAIMS 1-9 ARE UNPATENTABLE UNDER 35 U.S.C. § 103(A) AS OBVIOUS OVER DREVON

166. It is my opinion that the cited sections in **Table 8** below, which are also included in the IPR Petition, establish that the disclosures of Drevon in light of inherent functionality of a lipase on a component of a fingerprint renders Claims 1-9 obvious.

1. Independent Claim 1

167. Drevon discloses enzyme immobilization into polymers and coatings (Drevon [Ex. 1003] at pg. 3:Abstract; pg. 77:ln. 5-12, pg. 19;ln. 16 to pg. 20:ln. 3) and that immobilization refers to the preparation of insoluble biocatalytic derivatives and involves the coupling of enzymes to solid supports that are either organic or inorganic (*Id.* at pg. 18:ln. 7-8).

168. Drevon discloses that such enzyme can be a lipase that retains enzymatic activity once immobilized. (*Id.* at pg. 79:ln. 7-14; Table 1: Lipase; pg. 214:1-4) and that the development of coatings or films with biocatalytic

properties is of major interest for antifouling. (*Id.* at pg. 74:ln. 15-17).

169. Drevon discloses solid supports and coatings to which enzymes are coupled such as by immobilization. (*Id.* at pg. 18:ln. 7-8; pg. 19:ln. 5-19; pg. 20:ln.1-3; pg. 70:ln. 6-9; pg. 77:ln. 5-12; pg. 169:3-8).

170. Drevon discloses associating enzymes with coatings and polymer films (*Id.* at pg. 77:ln. 5-12; pg. 19;ln. 16 to pg. 20:ln. 3), immobilizing enzymes by coupling them to solid supports (*Id.* at pg. 18:ln. 7-8; pg. 70:ln. 1-9; pg. 169:3-6), and that at least one of the enzymes can be a lipase that retains enzymatic activity once immobilized (*Id.* at pg. 79:ln. 7-14; Table 1: Lipase; pg. 214:1-4).

171. Although Drevon does not explicitly disclose facilitating the removal of a fingerprint by vaporization from the lipase associated substrate or coating when contacted by a fingerprint, Drevon discloses that the development of coatings or films with biocatalytic properties is of major interest for antifouling (*Id.* at pg. 74:ln. 15-17).

172. It is my opinion that, at the time of the invention of the '618 Patent, a POSITA would have appreciated that an enzyme immobilized solid support as disclosed by Drevon is capable of facilitating the removal of a bioorganic stain (i.e., a fingerprint) by vaporization when such enzyme is the disclosed lipase and such lipase immobilized solid support is present in an environment

that supports vaporization of the enzymatically degraded component(s) of the bioorganic stain

173. Thus, it is my opinion that the Drevon in light of inherent functionality of a lipase enzyme on components of a "fingerprint" renders obvious the invention as recited in Claim 1, as indicated through the respective citations in Table 8 below.

2. Dependent Claim 2

174. Drevon discloses that enzymes can be immobilized on a solid support by covalent attachment. (*Id.* at pg. 18:ln. 13-15; pg. 57:ln. 18-19; pg. 58:ln. 4-21).

3. Dependent Claim 3

175. Drevon discloses that enzymes can be coupled to a solid support by non-covalent adherence (*Id.* at pg. 56:ln. 11-pg. 57:ln. 1; pg. 76:ln. 11-13; pg. 18:ln.1-4) and can be admixed into a coating that is provided on a solid support (*Id.* at pg. 76:ln. 14- pg. 77:ln. 4).

4. Dependent Claims 4

176. Drevon discloses heating of the enzyme immobilized solid support (*Id.* at pg. 18:ln. 18-20; pg. 58:ln. 13-15).

5. Dependent Claim 5

177. Although Drevon discloses heating of the enzyme immobilized solid support (*Id.* at pg. 18:ln. 18-20; pg. 58:ln. 13-15), and discloses enzymes immobilized in coating polymers at differing temperatures for periods greater than 30 minutes (*Id.* at Table 2: pg. 98:OPH, Pronase, ln. 18-20; pg. 58:ln. 13-15), Drevon does not explicitly disclose said heating of lipase is for at least 30 minutes.

178. It is my opinion, however, that at the time the invention of the '618 Patent was made, it would have been well-known to a POSITA that consumer products such as cell phones, touch-screens of devices, door handles of automobiles, and the like were subject to frequent contact with hands and fingers and that residue of fingerprints often leave unpleasant marks (i.e., bioorganic stains) on the surface. (see '618 Patent 1:16-20; 3:1-8)

179. It is also my opinion that it would have also been well-known to a POSITA at the time the invention of the '618 Patent was made that substrate surfaces and coating surfaces of such consumer products that are exposed to bioorganic stains are routinely subjected to heating for at least 30 minutes during their routine use (e.g., an automobile being exposed to sunlight, electrical powering of cell phone electrical components, charging of batteries of a cell phone, electrical powering of

a device having a touchscreen, outdoor furniture and the like being exposed to sunlight and elevated ambient temperatures, and the like).

6. Dependent Claim 6

180. Drevon discloses that the solid support material can be a polyurethane resin (*Id.* at pg. 70:ln. 5-11; pg. 106:ln. 5-13; pg. 68:ln. 5-11; pg. 169:3-6) or an acrylic resin or acrylate polymer coating (*Id.* at pg. 77:ln. 5-9; pg. 101:ln. 13-17) or polyacrylate (*Id.* at pg. 169:3-6).

181. Drevon teaches cross-linking of a polyurethane coating (*Id.* at pg. 106:5-10).

182. It is my opinion that polyurethane resin, an acrylic resin, an acrylate polymer coating, polyacrylate are each well known to be an organic crosslinkable polymer resin.

7. Dependent Claim 7

183. Drevon discloses that organic crosslinkable materials from which the solid support or coating is made can comprise a functional group (*Id.* at pg. 58:ln. 4-10) including at least one of amine, hydroxyl, and isocyanate. (*Id.* at pg. pg. 68:ln. 5-13; pg. 70:ln. 9-20; pg. 101:ln. 2-16; pg. 106:ln. 5-10). In view of these disclosures of Drevon, a POSITA would have found it obvious at the time of the invention of the '618 Patent was made that the solid support to which the disclosed lipase enzyme is immobilized can be

an organic crosslinkable material that comprises a functional group including at least one of amine, hydroxyl, and isocyanate.

8. Dependent Claim 8

184. Drevon discloses that solid supports upon which an enzyme such as the disclosed lipase enzyme can be immobilized can be made from polyacrylate and polyurethane (*Id.* at pg. 68:ln. 5-13; pg. 70:ln. 9-20; pg. 169:ln. 3-6) and coatings to which enzymes can be immobilized can be polyurethane. (*Id.* at pg. 101:ln. 2-16)

9. Dependent Claim 9

185. Drevon discloses that polymers such as polystyrene, polyacrylate, polymethacrylate, and polyurethanes have been shown to be viable matrices for the irreversible and multi-point immobilization of enzymes. (*Id.* at pg. 169:ln. 3-6).

186. Drevon discloses hydroxyl functional groups in the context of a polyol cross-linking with polyisocyanates to produce an enzyme polyurethane coating and similar approach for enzyme acrylate polymer coating. (*Id.* at pg. 101:ln. 9-17, pg. 106:ln. 5-10).

187. It is my opinion that, in view of these disclosures of Drevon, a POSITA would have found it obvious at the time the invention of the '618 Patent

was made that the solid support to which the disclosed lipase enzyme is immobilized can be a hydroxyl-functionalized acrylate resin.

188. Thus, it is therefore my opinion that the disclosures of Drevon render claims 1-9 obvious and unpatentable.

189. Table 8 – Claims 1-9 Mapping Based on Drevon

1. [P1] A method of facilitating the removal of a fingerprint on a substrate or a coating comprising:	Drevon at pg. 3:Abstract; pg. 77:ln. 5-12, pg. 19;ln. 16 to pg. 20:ln. 3; pg. 18:ln. 7-8; pg. 79:ln. 7-14; Table 1: Lipase; pg. 214:1-4; pg. 74:ln. 15-17 See also sections IV. D and IV.E of the Petition.
Element [A1] providing a substrate or a coating;	Drevon at pg. 18:ln. 7-8; pg. 19:ln. 5-19; pg. 20:ln.1-3; pg. 70:ln. 6-9; pg. 77:ln. 5-12; pg. 169:3-8
Element [B1] associating a lipase with said substrate or said coating such that said lipase is capable of enzymatically degrading a component of a fingerprint,	Drevon at pg. 77:ln. 5-12; pg. 19;ln. 16 to pg. 20:ln. 3; pg. 18:ln. 7-8; pg. 70:ln. 1-9; pg. 169:3-6; pg. 79:ln. 7-14; Table 1: Lipase; pg. 214:1-4
Element [C1] facilitating the removal of a fingerprint by vaporization from the lipase associated substrate or coating when contacted by a fingerprint.	Drevon at pg. 74:ln. 15-17 See also sections IV. E, IV.F and IV.D of the Petition.
2. The method of claim 1 wherein said lipase is covalently attached to said substrate or to said coating.	Drevon at pg. 18:ln. 13-15; pg. 57:ln. 18-19; pg. 58:ln. 4-21
3. The method of claim 1 wherein said lipase is non-covalently adhered to or admixed into said substrate or said coating.	Drevon at pg. 56:ln. 11-pg. 57:ln. 1; pg. 76:ln. 11-13; pg. 18:ln.1-4; pg. 76:ln. 14- pg. 77:ln. 4
4. The method of claim 1 comprising heating said substrate or said coating	Drevon at pg. 18:ln. 18-20; pg. 58:ln. 13-15

or applying heat to a surface of said substrate or said coating subsequent to being contacted by a fingerprint.	
5. The method of claim 4 wherein said heating is for at least 30 minutes.	Drevon at pg. 18:ln. 18-20; pg. 58:ln. 13-15; Table 2: pg. 98:OPH, Pronase, ln. 18-20; pg. 58:ln. 13-15 See also the '618 Patent 1:16-20; 3:1-8
6. The method of claim 1 wherein said substrate or said coating comprises an organic crosslinkable polymer resin.	Drevon at pg. 70:ln. 5-11; pg. 106:ln. 5-13; pg. 68:ln. 5-11; pg. 169:3-6; pg. 77:ln. 5-9; pg. 101:ln. 13-17; pg. 169:3-6
7. The method of claim 6 wherein said organic crosslinkable polymer resin comprises a functional group of acetoacetate, acid, amine, carboxyl, epoxy, hydroxyl, isocyanate, silane, vinyl, or combinations thereof.	Drevon at pg. 58:ln. 4-10; pg. 68:ln. 5-13; pg. 70:ln. 9-20; pg. 101:ln. 2-16; pg. 106:ln. 5-10
8. The method of claim 6 wherein said organic crosslinkable polymer resin is aminoplasts, melamine formaldehydes, carbamates, polyurethanes, polyacrylates, epoxies, polycarbonates, alkyds, vinyls, polyamides, polyolefins, phenolic resins, polyesters, polysiloxanes, or combinations thereof.	Drevon at pg. 68:ln. 5-13; pg. 70:ln. 9-20; pg. 169:ln. 3-6; pg. 101:ln. 2-16
9. The method of claim 6 wherein said organic crosslinkable polymer is a hydroxyl-functionalized acrylate resin.	Drevon at pg. 169:ln. 3-6; pg. 101:ln. 9-17, pg. 106:ln. 5-10

H. GROUND 3B: CLAIMS 10 AND 11 ARE UNPATENTABLE UNDER 35 U.S.C. § 103(A) AS OBVIOUS OVER DREVON IN VIEW OF SCHNEIDER

190. It is my opinion that the cited sections in **Table 9** below, which are also included in the IPR Petition, establish that Drevon as applied above to Claim 1 in view of Schneider teaches all of the limitations in Claims 10 and 11 and render these claims obvious and unpatentable.

191. It is my opinion that a POSITA would have been motivated, or would have found it obvious, at the time that the invention was made to combine the disclosures of Drevon and Schneider because the disclosures of Drevon and Schneider are in overlapping technical fields, address similar technical disclosure relating to utilization and/or construction of articles subjected to microbial contamination, and present motivating and/or suggesting disclosure for such combination.

1. Dependent Claims 10 and 11

192. Although Drevon does not explicitly disclose that the enzyme is lipoprotein lipase, acylglycerol lipase, hormone-sensitive lipase, phospholipase Al, phospholipase A2, phospholipase C, phospholipase D, phosphoinositide phospholipase C, a lysophospholipase, or a galactolipase, Drevon discloses immobilizing enzymes by coupling them to solid supports and coatings (*Id.* at pg. 18:ln. 7-8; pg. 19:ln. 5-19; pg. 20:ln.1-3; pg. 70:ln. 6-9; pg. 77:ln. 5-12; pg. 169:3-8) and that at least one

of the enzymes can be a lipase that retains enzymatic activity once immobilize (*Id.* at pg. 79:ln. 7-14; Table 1: Lipase; pg. 214:1-4).

193. Schneider discloses "a coating composition comprising at least one enzyme, preferably an oxidase, capable of acting on a compound, such as a substrate for said oxidase, wherein said action results in the formation of an antifouling species including an antimicrobial species comprising an antimicrobial activity, and wherein said compound does not form part of said coating composition." (Schneider [Ex. 1004] at 0050:2-8).

194. Schneider further disclose that "the oxidase can be present in said coating composition in combination with one or more additional enzymes including, but not limited to, an esterase, including a lipase," (*Id.* at 0052:1-4) and that the lipase can be lipoprotein lipase (*Id.* at 0074:1-3) or triacylglycerol lipase (*Id.* at 0074:1-3).

195. It is my opinion that it would have been obvious to a POSITA at the time that the invention of the '618 Patent was made to modify the lipase of the lipase immobilized solid support disclosed by Drevon to be lipoprotein lipase or triacylglycerol lipase disclosed by Schneider.

196. It is my opinion that a motivation for such modification is the POSITA seeking enzymes the exhibit enzymatic activity against various lipids and both lipoprotein lipase and triacylglycerol lipase being well known to

enzymatically degrade components of bioorganic stains such as, for example lipids, fatty acids and the like (e.g., lipase disclosed by Drevon is well known to have comparable performance as lipoprotein lipase and triacylglycerol lipase with respect to enzymatically degrading lipid-based substances.)

197. It is my opinion that another motivation for such combination is that Drevon discloses that antifouling is an application for the enzyme immobilized solid supports thereof and Schneider analogously discloses enzyme coatings, including immobilized enzymes (*Id.* at 0110:5-12), for antifouling applications and, more specifically, antimicrobial applications. (*Id.* at 0050:2-8, 0052:1-4, 0074:1-3).

198. It is therefore my opinion that the combination of Drevon as applied above to Claim 1 in view of Schneider renders claims 10 and 11 obvious and unpatentable.

199. **Table 9 – Claims 10 and 11 Mapping Based on Drevon in view of Schneider**

10. The method of claim 1 wherein said lipase is lipoprotein lipase, acylglycerol lipase, hormone-sensitive lipase, phospholipase A1, phospholipase A2, phospholipase C, phospholipase D, phosphoinositide phospholipase C, a lysophospholipase, or a galactolipase.	Drevon at pg. 18:ln. 7-8; pg. 19:ln. 5-19; pg. 20:ln.1-3; pg. 70:ln. 6-9; pg. 77:ln. 5-12; pg. 169:3-8; pg. 79:ln. 7-14; Table 1: Lipase; pg. 214:1-4 Schneider at 0050:2-8; 0052:1-4; 0074:1-3; 0110:5-12

11. The method of claim 1 wherein said lipase is a triacylglycerol lipase.	Drevon at pg. 18:ln. 7-8; pg. 19:ln. 5-19; pg. 20:ln.1-3; pg. 70:ln. 6-9; pg. 77:ln. 5-12; pg. 169:3-8; pg. 79:ln. 7-14; Table 1: Lipase; pg. 214:1-4 Schneider at 0050:2-8; 0052:1-4; 0074:1-3; 0110:5-12

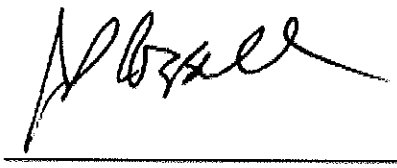
200. In signing this declaration, I recognize that the declaration will be filed as evidence in a contested case before the Patent Trial and Appeal Board the United States Patent and Trademark Office. I also recognize that I may be subject to cross-examination in this *Inter Partes* Review and that cross examination will take place within the United States. If cross examination is required of me, I will be available for any such cross examination.

201. I reserve the right to supplement my opinions in the future to respond to any arguments that the Patent Owner raises and to take into account new information as it becomes available to me.

202. I declare that all statements made herein of my own knowledge true and that

all statements made on information and belief are believed to be true, and further 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 or Title 18 of the United States Code.

Executed: September 28, 2016



Dr. David Rozzell
CEO and Founder
Sustainable Chemistry Solutions, Inc.

ATTACHMENT A: Résumé of Dr. David Rozzell

J. David Rozzell, PhD

Experienced consultant and executive in the life sciences, founder or co-founder of three companies, including BioCatalytics, Inc., a leading developer and producer of enzymes for pharmaceutical manufacturing and diagnostic use; and Sustainable Chemistry Solutions, Inc, a consultancy and provider of information products for the enzyme and biocatalysis industries and publisher of the Enzyme Industry Newsletter.

Tel: +1-818-388-6576 E-Mail: drozzell@provivi.com or jdrozzell@gmail.com

Education

University of Virginia	B.S.	1978	Chemistry
Harvard University	Ph.D.	1983	Chemistry
Harvard Business School	Summer Business Program, 1982		

Positions and Employment

Genetics Institute, Inc., Cambridge, MA
1983-1986; Senior Scientist
1986-1988; Director of Biocatalysis Research
Built and managed an interdisciplinary group reaching the size of 8 professionals. Directed the research and development activities of the Applied Enzymology and Biocatalysis groups. Efforts resulted in more than $1 million in revenues through funding and license agreement, and the commercialization of processes to manufacture optically active amino acids at the multi-hundred ton per year scale.

Celgene Corporation, Warren, NJ
1988-1991; Director of Research and Biotreatment Systems
Responsibility for directing both proprietary and collaborative research programs focused on the production of pharmaceutical intermediates and specialty chemicals. Technology employed a combination of biocatalytic reactions and organic chemistry.

Exogene Corporation, Monrovia, CA
1991-1992; Vice-President of Research & Development
1992-1994; President
Responsible for business development, negotiation of sponsored research and technology licensing agreements, general scientific guidance of the company's research, and supervision of the administrative and senior scientific staff.

EraGen Biosciences, Inc., Madison, WI

1994-1996: Founder and Acting CEO
Co-founded a start-up biotechnology company focused on applications of non-standard nucleic acid bases and protein structure prediction. Raised seed capital from individual investors and the Novartis Venture Fund. Acted as CEO until full-time person was recruited to establish the company in its first headquarters in Florida.

BioCatalytics, Inc., Pasadena, CA
1996-July 2007: Founder, President, CSO and CEO
Established a biotechnology company to develop and commercialize enzymes and enzyme-based processes for the production of optically active pharmaceutical intermediates and other specialty chemicals. Built company into a profitable seller of novel enzymes for chemical synthesis, with the world's largest enzyme product line. Established a European office in 2005 and a subsidiary BioCatalytics Europe GmbH in Graz, Austria in 2006. BioCatalytics, Inc. was acquired in July 2007 by Codexis, Inc.

Codexis, Inc., Pasadena, CA
July 2007-Oct 2008, VP, Biocatalysis Technology and Applications
Joined Codexis, Inc, as VP following acquisition of BioCatalytics by Codexis, Inc. Responsible for the identification and development of new technologies, including technologies developed and in-licensed through external collaborations. Managed a network of external collaborations in the USA and Europe. Promoted the company and supported business development activities through technical presentations, press conferences, and written articles. Initiated an emphasis on Green Chemistry.

Solidus Biosciences, Inc., San Francisco, CA
March 2009-October 2010, President & CEO
Hired as President and CEO to lead a company with a novel, chip-based *in vitro* toxicology platform. Responsible for managing company operations, setting business strategy, developing new customer relationships, and raising funds from investors.

Sustainable Chemistry Solutions, Inc., Burbank, CA
January 2012-2015, Founder and CEO
Publisher, founder, and owner of the web site http://www.bio-catalyst.com focusing on providing information and insights on biofuels, bio-based chemicals, and biocatalysis. Publisher of monthly newsletter *Enzyme Industry Newsletter*. Offer information products and consulting services related to enzymes and biocatalysis to pharmaceutical and chemical companies. Provide consulting support to programs for the development of novel enzymes and their applications, and also for pathways in bio-based chemical production. Creator and publisher of the Enzyme Company Guide and the Biocatalysis Enzyme Guide, providing business and technical information to industry specialists. Also offer expert witness services in patent litigation and cases involving enzymes and the development and enzyme-based processes and applications.

Catylix, Inc., Burbank, CA

February 2011-2015, CEO and Co-Founder with Professor John F. Hartwig
Established company to develop and commercialize a novel, broadly-useful chemistry for adding fluorine-containing functional groups to chemical compounds. First product launched in July 2011. Main product applications are in the discovery of pharmaceuticals and crop protection agents with improved efficacy and metabolic stability.

Provivi, Inc. Santa Monica, CA
August 2015-Present, Sr. Vice-President, Biocatalysis
Joined Provivi, Inc., an early-stage biotechnology company, to lead development and commercialization of novel enzymes catalyzing the synthesis of chiral cyclopropanes via a carbene transfer mechanism. Responsibilities include managing internal R & D, business development, customer acquisition and project management to meet rigorous timelines for development.

Other Work Experience

Consultant and Member of Scientific Advisory Board
Wella AG, Darmstadt, Germany
Served as member of scientific advisory board and provided consulting assistance to the biotechnology program established by Wella AG, the German Cosmetic and hair care company. Inventor on two patents for the coloration and modification of the properties of hair using enzymes. Wella AG was acquired by Procter & Gamble in 2003.

Advisory Board Member, Eucodis Biosciences, Vienna, Austria (2011-2013)

Consultant and Scientific Advisor, Almac Sciences, Craigavon, United Kingdom (2010 to present)

Organizer of International Symposium "Development and Application of enzymes in Biotechnology" sponsored by Informa Group and held in Duesseldorf, Germany, April 14-15, 2015

Honors
Co-Chair, Gordon Conference on Biocatalysis, 1992
Chairman and Organizer and Speaker at various international symposia on enzymes and biocatalysis
Member of Editorial Board, Journal of Industrial Microbiology
Member of Industrial Advisory Board, Journal of Advanced Synthesis and Catalysis
Member of Board of Directors, EraGen Biosciences, Inc., Madison, WI
Member of Board of Directors, Pasadena Bioscience Center
Member of Advisory Board, Los Angeles-Orange County Biotechnology Center
Member, Blue Ribbon Steering Committee, Life Sciences Summit for Southern California

Selected Publications

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4. "Combining Enzymatic and Chemical Steps in the Synthesis of Biologically Valuable Compounds: Isotopically Chiral Methyl Acetate": Steven A. Benner and J. David Rozzell; J. Org. Chem., 48, 1190-93 (1983).
5. "Stereochemical Imperative in Enzymic Decarboxylations. Stereochemical Course of the Decarboxylation Catalyzed by Acetoacetate Decarboxylase": J. David Rozzell and Steven A. Benner; J. Am. Chem. Soc., 106, 4937-41 (1984).
6. "Enzymatic Catalysis in Organic Media": J. S. Deetz and J. David Rozzell; The World Biotech Report 1986, 2, 25-33 (1986).
7. "Immobilized Aminotransferases for Amino Acid Production": J. David Rozzell; Methods in Enzymology, 136, 479-97 (1987).
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9. "Enzyme-Catalyzed Reactions in Non-Aqueous Media": Jeffrey S. Deetz and J. David Rozzell; Trends in Biotechnology, 6, 15-19 (1988).
10. "Enzymatic Catalysis in Non-Aqueous Media": Jeffrey S. Deetz and J. David Rozzell; Annals of the New York Academy of Sciences, 230-234 (1988).
11. "Production of Cyclodextrins Using Immobilized Enzymes": Steven P. Crump and J. David Rozzell; Proceedings of the 4th International Symposium on Cyclodextrins, 47-53, Kluwer Academic Publishers (1988).
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15. "Pathway for the Metabolism of Terephthalic Acid By Pseudomonas": Mark E. Ruppen and J. David Rozzell; Proceedings of the 5th European Congress on Biotechnology (1990).
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17. "The Production of Amino Acids by Transamination": J. David Rozzell; in Biocatalytic Production of Amino Acids and Derivatives: New Developments and Process Considerations, Hanser Publishers (1992).
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Books

Editor of book "Biocatalytic Production of Amino Acids and Derivatives," Hanser Publishers, Munich, 1992.

“Enzyme Sources Guide” published by Sustainable Chemistry Solutions, Inc., Burbank, 2014.

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“Biocatalysis Company Guide” published by Sustainable Chemistry Solutions, Inc., Burbank, 2015.

Patents

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16. US 6,830,904: Method for Producing Diastereomers of Isoleucine (2004).
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19. US 7,081,535: Hydroxy-Amino Acids (2006).
20. US 7,156,884: Compositions and Methods for Dyeing Keratinous Fibers (2007).
21. US 7,202,070: Method for the Reductive Amination of a Ketone Using a Mutated Enzyme (2007).
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Various Patent Applications Pending

Research Support

SBIR AI45241: Production of Non-Naturally Occurring Amino Acids; Phase 1 and 2; March 1999- Feb. 2002. PI. Development of novel methods to produce single

enantiomers of non-naturally occurring amino acids using transaminase enzymes that are newly discovered or created by directed evolution.

SBIR GM65675: Novel Enzymatic Reductive Amination; Phase 1; May 2002-Dec 2002. PI. Creation new enzymes for reductive amination of ketones by laboratory enzyme evolution and high-throughput screening.

SBIR GM59541: Salt-Activated Enzymes for Organic Synthesis; Phase 1; April 1999-Sept. 1999. PI. Evaluation of a novel method of salt-immobilization to activate enzymes for use in organic solvents.

SBIR DK55951: Method for Neonatal Screening for Homocystinuria; Phase 1 and 2; Sept. 1999-Jan. 2003. PI. Development of an enzyme-based method to quantitate homocysteine in blood or urine samples of newborns.

SBIR GM60822: Production of Chiral Aminoalcohols; Phase 1 and 2; Jan. 2000-Aug. 2003. PI. Development of a novel chemo-enzymatic method for the production of single diastereomers of chiral amino alcohols for use as pharmaceutical intermediates.

SBIR GM067536: Evolving Improved Formate Dehydrogenases: Phase 2: Sept. 2004-Aug. 2006. PI. Creation of new formate dehydrogenase enzymes having improved rate, stability, and activity on NADP+ as well as NAD+. Applications are for the recycle of nicotinamide cofactors in the synthesis of chiral compounds through redox processes.

ATTACHMENT B: "*Immobilization of Enzymes by Covalent Attachment.*" Chapter 20 in "Methods in Biotechnology, Vol. 17: Microbial Enzymes and Biotransformations," Ed. J. L. Barredo, Humana Press, Inc. Totowa, NJ, 2005.

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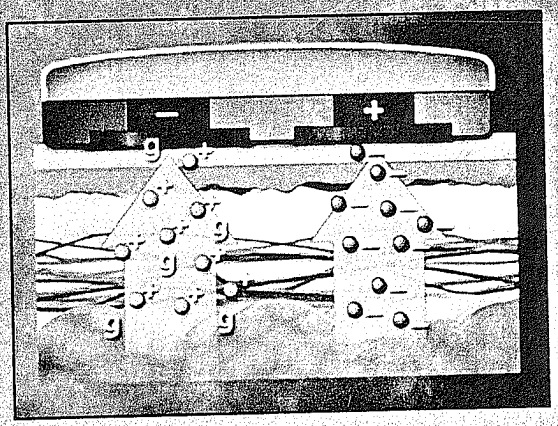
Edited by

José Luis Barredo



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METHODS IN BIOTECHNOLOGY™

Microbial Enzymes and Biotransformations

Edited by

José Luis Barredo

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Immobilization of Enzymes by Covalent Attachment

Scott J. Novick and J. David Rozzell

Summary

Enzymes are finding increasing use for the production of agrochemicals, pharmaceuticals, and fine chemicals. They are almost always used in the immobilized form in order to simplify their removal from the product stream. In addition, immobilization often enhances the stability of the enzyme. Immobilization can be performed in a number of ways. This chapter discusses various methods, properties, and uses of covalently immobilized enzymes.

Key Words: Immobilized enzymes; covalent immobilization.

1. Introduction

1.1. Historical Perspective

An immobilized enzyme is generally defined as "the imprisonment of an enzyme molecule in a distinct phase that allows exchange with, but is separated from, the bulk phase in which substrate effector or inhibitor molecules are dispersed and monitored" *(1)*. Immobilized enzyme technology dates back to the 1910s to the 1930s, when proteins were physically adsorbed onto surfaces such as charcoal, kaolinite, cellulose, and glass beads *(2–4)*. But it was not until the 1950s and '60s with the work of Katchalski-Katzir, and Chibata and coworkers that real advancements were beginning to be made in the development and applications of immobilized enzyme materials *(5)*. This early work culminated in the First Enzyme Engineering Conference in 1971. The first industrial use of immobilized enzymes was for amino acid production. Chibata and coworkers at Tanabe Seiyaku (Japan) in 1969 used an immobilized L-aminoacylase in a packed bed reactor to resolve various DL-amino acids into their enantiomerically pure forms. Since that time, immobilized enzymes have become increasingly important for the production of many important chiral compounds (i.e., amines and alcohols) for the pharmaceutical and fine chemical industries.

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Table 1
Stabilization Effects Immobilization Imparts to Enzymes *(8)*

1. Prevention of either proteolysis or aggregation by spatial fixation of enzyme molecules to the support.
2. Unfolding of the enzyme is reduced due to multipoint covalent or adsorptive attachment to the support, and/or intramolecular crosslinking of the enzyme.
3. Multimeric enzymes would have a lower likelihood to dissociate if all subunits are attached to the support.
4. Denaturing agents (e.g., chemical inactivators) can be excluded from the enzyme by the support or inactivated by the support before reaching the enzyme (e.g., decomposition of hydrogen peroxide, produced during the oxidation of glucose by glucose oxidase, catalyzed by activated carbon).
5. Shifting by a charged support of the local pH, thus preventing pH inactivation of the enzyme.
6. Exclusion by the support (e.g., an encapsulation membrane) of proteases from the enzyme's environment.
7. Increased thermal stability due to multipoint attachment of enzyme to support.

1.2. Reasons for Enzyme Immobilization

The principal advantage of immobilizing enzymes is to retain the catalyst in the reactor. This can greatly improve the economics of a process. For a continuous process, a soluble enzyme would be washed out of the reactor along with the product stream. A process like this would not be economically feasible if the biocatalyst is very expensive (as is often the case) and cannot be reused. Although an ultrafiltration setup could be used to retain the enzyme, it is often too costly, both in capital and operation, on a large scale. Also, having a soluble enzyme in the product would not be desirable if the biocatalyst can cause the product to undergo side reactions or if there are toxicity effects associated with the catalyst, as will often be the case if the product is an intravenous drug *(6)*. Another advantage of immobilizing enzymes is to increase enzyme activity or stability especially under denaturing conditions *(7,8)*. Thermal stability can often be improved by many orders of magnitude compared to the soluble enzyme *(9–11)*. Activity of an enzyme in nonaqueous media can also be significantly higher than the native enzyme *(12–18)*. Another important advantage is the ability to control the microenvironment of the immobilized enzyme. For example, by immobilizing an enzyme on an acidic support (such as poly [acrylic acid]), the catalyst can be used at higher pHs, where the substrate may be more soluble, while the pH of the microenvironment surrounding the enzyme could be much closer to the enzyme's optimum pH. These and other stabilizing effects of immobilization are listed in **Table 1.**

There are also limitations to immobilizing enzymes. Some inherent catalytic activity is almost always lost during the immobilization procedure. Enzymes possess highly defined, yet relatively fragile three-dimensional structures that must come in contact and interact with the rigid support. These binding forces, such as covalent bonds or adsorptive interactions, are often more powerful than the secondary forces, such as hydrogen bonding and hydrophobic and ionic interactions, which hold proteins in their proper configuration for enzymatic activity. In addition, no covalent immobilization method is able to bind only the nonessential elements of every enzyme (if they even exist) to the support, and all supports create asymmetric force fields and change the water activity around the biocatalyst *(6)*. In addition, apparent activity can be decreased by mass transfer limitations. However, the increase in stability and ease of removal from the product stream and reuse often more than make up for any decrease in activity.

1.3. Enzyme Immobilization Methods

In general five techniques have been described for immobilization of enzymes. It is important to point out that there is no one universal immobilization system; instead, a range of methodologies must be evaluated depending on the enzyme to be immobilized and the overall process in which the immobilized enzyme is to be used. Also, most immobilization methods, although conceptually distinct, often overlap to a certain extent, and in some cases, multiple immobilization methods are employed.

One of the simplest and most economical immobilization methods is adsorbing an enzyme onto a support. The enzyme is bound to the support via ionic or nonionic interactions. Supports often include carbohydrate-based or synthetic polymer ion-exchange resins or uncharged supports such as polymers, glasses, and ceramics. The main drawback of this method is the leaching of enzyme from the support.

Cross-linking enzyme molecules with themselves, or more often with an inert protein such as gelatin or bovine serum albumin, results in an insoluble active enzyme preparation that can be readily handled or manipulated in a continuous reactor. Glutaraldehyde, adipimate esters, and diisocyanates are often used as the cross-linking agent. Significant inactivation of the enzyme may result during the cross-linking step and is the major drawback of this method.

Entrapment of an enzyme within a polymeric matrix is another method used for enzyme immobilization. This is often done by mixing the enzyme with a monomer and a cross-linker, and polymerizing the monomer around the enzyme. Leaching of the enzyme out of the matrix and mass transfer limitations of substrate diffusing into the matrix can limit the use of this technique.

Encapsulating or confining an enzyme within a membrane is another method for enzyme immobilization. Ultrafiltration membranes or hollow fibers made of

polyethersulfone, cellulose nitrate or acetate, or nylon are often used. The pore size must be properly chosen to allow substrate and product to enter and exit the membrane while still retaining the enzyme. Since the enzyme exists in its soluble form, activity is usually high. Membrane fouling and reduced flow rates are drawback of this technique.

The fifth immobilization method, covalent attachment of enzymes to a support, will be the subject of the rest of this chapter.

1.4. Covalent Enzyme Immobilization

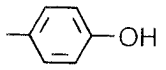
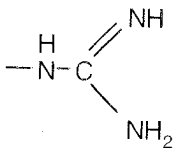
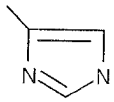
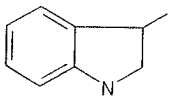
Covalent attachment of enzymes to an insoluble support is an often-used method of enzyme immobilization. It is especially useful when leaching of enzyme from the support is a concern. The enzyme is usually anchored via multiple points and this generally imparts greater thermal, pH, ionic strength, and organic solvent stability onto the enzyme since it is more rigid and less susceptible to denaturation. Covalently immobilized enzymes are also often more resistant to degradation by proteolysis.

There are, however, some drawbacks to covalent enzyme immobilization. Typically it is more expensive and complex to covalently immobilize an enzyme compared to the other methods due to the higher costs of the support. The support often needs to be activated prior to immobilization. The increased stability and typically minimal enzyme leaching often more than make up for these shortcomings.

Enzymes contain a number of functional groups capable of covalently binding to supports. **Table 2** lists these groups along with their relative frequency in a typical protein *(19–21)*. Of the functional groups of enzymes listed, $-NH_2$, $-CO_2H$, and $-SH$ are most frequently involved in covalent immobilization. Amines and sulfhydryls are good nucleophiles, while the ability to activate carboxylates so they are reactive toward nucleophiles makes these groups important as well. The phenolic ring of tyrosine is also extremely reactive in diazo-coupling reactions, and its hydroxyl group can be an excellent nucleophile at basic pH. Aldehydes can react with the guanidino group of arginine and, although histidine displays a lower nucleophilicity, it can react in some cases with supports activated with tosylates, tresylates, or other good leaving groups.

The supports to which the enzymes are attached to can vary greatly. They can be either natural polymers, such as modified cellulose, starch, dextran, agal polysaccharides, collagen, and gelatin; or they can be synthetic, such as polystyrene, polyacrylamide, polyacrylates, hydroxyalkyl methacrylates, and polyamides. Inorganic supports can also be used, such as porous glass, metal oxides, metals, sand, charcoal, and porous ceramics. The variety of chemistries available for covalent attachment allows the conditions of immobilization to be tailored to

Table 2
Reactive Functional Groups in Enzymes and Their Average Occurrence in a Typical Protein *(19–21)*

Structure of reactive group	Reactive group	Occurrence in average protein
$-NH_2$	ε-Amino of lysine and N-terminus	5.9
$-CO_2H$	Carboxylate of glutamic acid, aspartic acid, and C-terminus	6.3 (Glu), 5.3 (Asp)
$-SH$	Thiol of cysteine	1.9
	Phenolic of tyrosine	3.2
	Guanidino of arginine	5.1
	Imidazole of histidine	2.3
$-S-S-$	Disulfide of cystine	—
	Indole of tryptophan	1.4
$-CH_3-S-$	Thioether of methionine	2.2
$-CH_2OH$	Hydroxyl of serine and threonine	6.8 (Ser), 5.9 (Thr)

each enzyme system. This also allows the microenvironment of the enzyme to be tailored by appropriate modification of the support surface; hydrophobic moieties or ionically charged groups may be used to alter the support to enhance the enzyme-catalyzed reaction of interest. Some supports, such as those containing

Fig. 1. Enzyme immobilization onto hydroxyl containing supports via activation with cyanogen bromide (top) or *S*-triazine derivatives (bottom).

epoxide groups can be used directly for enzyme binding. However, most supports require preactivation before enzymes are able to bind to it. The following sections describe some typical covalent attachment methodologies.

1.4.1. Covalent Attachment Onto Polyhydroxyl Supports

Polyhydroxyl supports, such as porous glass, and especially polysaccharides are among the most commonly used matrices for enzyme immobilization. Because hydroxyl groups are poor leaving groups they must first be activated. This is typically done with cyanogen bromide *(22)*. Other activating agents such as *S*-triazine derivatives have also been used. Once the support is activated it is able to covalently couple to an enzyme usually through the ε-amino group of lysine or through the amino terminus. The mechanism of derivatization polyhydroxyl supports with the above two derivatizing agents and the subsequent enzyme immobilization is shown in **Fig. 1.**

Supports that have been preactivated with cyanogen bromide can be stored for periods of up to 1 yr at freezer temperatures. Preactivated supports are also available commercially. Once the support is activated, coupling of the enzyme requires no more than exposing the enzyme to the activated support in an aqueous solution for a few hours, followed by extensive washing to remove any protein that is not covalently bound.

This method is extremely popular in the lab scale; however, it has not been widely used in large-scale applications. The activating agent, cyanogen bromide, is extremely toxic, and most carbohydrate supports, such as cellulose, agarose, and dextran, have poor mechanical stability compared to other support materials. Also, since the supports are natural polysaccharides, microbial contamination and degradation are a concern. Finally, the bond between the enzyme and the support is potentially susceptible to hydrolytic cleavage, which would cause leaching of the enzyme from the support over time.

1.4.2. Covalent Attachment onto Carboxylic Acid-Bearing Supports

Carboxylic acid-containing supports, such as copolymers of (meth)acrylic acids with (meth)acrylic esters have also been used as an immobilization support. These must also be activated and this is usually done with a carbodiimide reagent. Under slightly acidic conditions (pH 4.75–5.0) carbodiimides react with carboxylic acid groups to give the highly reactive *O*-acylisourea derivatives. The supports are then washed to remove excess reagent and the enzyme is coupled to the activated support at neutral pH to give stable amide, thioester, or ester linkages, depending on the residue reacting with the support. The most widely used water-soluble carbodiimides are 1-ethyl-3-(3-dimethylamino propyl)-carbodiimide (EDC) and 1-cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide (CMC), both of which are available commercially. The reaction

Fig. 2. Activation of a carboxylic acid containing support with a carbodiimide followed by enzyme coupling.

Fig. 3. Activation of an amine-bearing support with glutaraldehyde followed by enzyme coupling.

scheme for of activating a carboxylic acid-containing support and subsequent enzyme coupling is shown in **Fig. 2.**

1.4.3. Covalent Attachment Onto Amine-Bearing Supports

Amine-bearing supports are among the most used and the most useful supports for covalent enzyme immobilization. These supports can either be organic or inorganic supports bearing amine functionality. The most frequent technique for introducing amine groups on inorganic supports is via aminosilane attachment *(23–25)*. For example, 3-aminopropyltriethoxysilane can be coupled to porous glass to give pendent amine groups *(26)*. This silane has been developed through the pioneering work at Corning Glass Works *(23)*.

Another common amine-bearing support is polyethyleneimine-coated particles. Polyethyleneimine is a common polyamine derived from the polymerization of ethyleneimine to give highly branched polymers containing approximately 25% primary amines, 25% tertiary amines, and 50% secondary amines. This polymer can be coated onto various supports including alumina *(27)*, carbon *(28)*, diatomaceous earth *(29)*, and polyvinyl chloride-silica composites *(30,31)*.

The coupling of an enzyme to amine-bearing supports can be done in a number of ways. The most common way is through the use of difunctional reagents, such as diimidate esters, diisocyanates, and dialdehydes. Glutaraldehyde is often used, as it is one of the least expensive difunctional reagents available in bulk. This reagent reacts in a complex fashion to form Schiff bases with amine groups on the support and produces pendent aldehydes and α,β-unsaturated carbonyl functionalities through which enzymes may attach. Enzyme attachment is accomplished simply by mixing the enzyme with the activated support. A simplified example of this is shown in **Fig. 3.** The acid-labile Schiff bases can be reduced to more stable secondary amine bonds with sodium borohydride to increase the stability of the enzyme-support linkage.

Crump and coworkers *(32)* have described the immobilization of an L-amino acid transaminase onto a polyethyleneimine coated PVC-silica support matrix that was activated with glutaraldehyde. Very high binding efficiency and residual activity were obtained. After washing, 93% of the enzyme offered was bound to the support (total loading was about 10%) and the enzyme retained approximately 89% of the soluble activity. Both these values are unusually high for immobilized enzymes, but not necessarily atypical for this type of support and immobilization chemistry.

Enzymes can also be covalently bonded directly to amine-bearing supports via the enzyme's carboxyl groups. These must first be activated with a carbodiimide or similar reagent prior to immobilization. The activation step can cause enzyme inactivation and thus this method is not used as often.

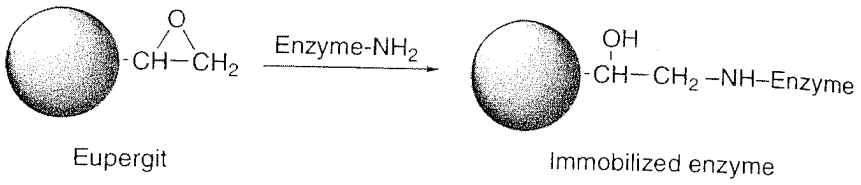


Fig. 4. Enzyme immobilization to Eupergit via free amino groups.

Diisocyanates have also been used as a coupling agent between amine-bearing supports and enzymes *(33)*. If alkaline conditions are used a substituted urea bond is formed between an amine on the enzyme and the isocyanate. If moderately acidic conditions are employed, the isocyanate will react with a hydroxyl group on the enzyme and form a urethane bond. Isothiocyanates have also been used successfully *(23)*.

Another amine-bearing support, developed by Leuta and coworkers *(34)*, is mineral or carbon particles coated with chitosan. Chitosan is deacylated chitin, a polymer of glucosamine, and contains an available amino group for chemical activation and enzyme binding using methods similar to those described for the other amine-bearing supports.

1.4.4. Covalent Attachment to Reactive Polymer Supports

Due to the preactivated nature of epoxy-containing supports, these materials have gained considerable attention as commercially useful support matricies for enzyme immobilization. A commercial epoxy-containing support is available from Röhm Pharma Polymers (Piscataway, NJ) under the trade name Eupergit. The material is a crosslinked copolymer of methacrylamide and oxirane containing monomers and consists of spherical beads of about 200 μm in diameter. Eupergit is available in two varieties, Eupergit C and Eupergit C 250 L, with their differences being their oxirane content and pore size. Eupergit C has average pore radius of 10 nm and an oxirane content of 600 μmol/g, while Eupergit C 250 L has a pore size and oxirane content of 100 nm and 300 μmol/g, respectively *(35)*. Eupergit C 250 L is targeted for the immobilization of large molecular weight enzymes (>100 kDa). Immobilization of enzymes to Eupergit is relatively simple. The enzyme solution is brought in contact with the Eupergit beads either quiescently or with slight mixing (magnetic stirbars should be avoided to prevent fractionation of the beads) for 24–96 h. This can be done either at room temperature, or if the enzyme is unstable, 4°C will also work. Various pHs can be used for the binding. Under neutral and alkaline conditions the amino groups on the enzyme are principally responsible for binding to the support (**Fig. 4**). Under acidic and neutral conditions sulfhydryl and carboxyl groups take part in binding.

Immobilization to Eupergit does not change the charged state of the enzyme. Typically, it is best to bind the enzyme to the support at the pH at which activity is optimum for the enzyme. The various parameters mentioned above—mixing type, immobilization time, temperature, pH, and also ionic strength (0.5–1 *M* buffer or neutral salt is often optimal)—can be varied to optimize the amount of enzyme immobilized and the residual activity. Once the enzyme is bound to the support, the binding is stable over the long term and it is stable over a wide pH range, from 1.0 to 12.0. Also, because Eupergit is electrically neutral, pH changes do not effect the swelling of the gel.

After the enzyme has been bound, typically only about 1% of the available epoxy group are involved in enzyme immobilization. The remaining groups will slowly hydrolyze into diols or they can be quenched with a variety of compounds that can effect the microenvironment around the immobilized enzyme by making it more hydrophilic, hydrophobic, or charged. This in turn can effect the stability or activity of the bound enzyme. Bovine serum albumin, dithiothreitol, Tris-buffer, mercaptoethanol, various amino acids (i.e., lysine or glycine), and ethanolamine are among some of the quenching reagents that have been used, and in many cases activity of the immobilized enzyme can be altered depending on the quenching reagent.

There have been two extensive reviews recently published concerning the immobilization of enzymes to Eupergit *(35,36)*. In these reviews, the details of the immobilization of nearly two dozen different enzymes are presented. In addition to Eupergit, other epoxy-containing polymers have been investigated for the covalent attachment of enzymes *(37–42)*.

Polyacrolein beads is another useful reactive-polymer carrier for covalent enzyme immobilization. Margel *(43)* synthesized such beads and encapsulated them into agarose prior to enzyme binding. Because these supports are polyaldehydes, enzymes are bound in a similar way as with glutaraldehyde activated supports. Various oligomers such as poly(lysine) and poly(glycine) have been attached to the polyacrolein beads to act as spacers between the particles and the enzyme. In both cases the poly(amino acids) are attached to the support through their terminal amino groups, or ε-amino groups in the case of poly(lysine), via Schiff bases (which can then be reduced). The enzyme is attached to the poly(lysine)-derivatized polyacrolein via the lysine ε-amino groups using glutaraldehyde as a linker. For the poly(glycine)-derivatized polyacrolein support, the terminal carboxyl group is activated with a water-soluble carbodiimide followed by enzyme binding. In some cases the use of these spacers has shown a significant increase in activity, especially for large-molecular-weight substrates. Covalent enzyme immobilization to paramagnetic polyacrolein beads has also been investigated *(44)*. Binding of enzymes to unmodified polyacrolein is shown in **Fig. 5.**

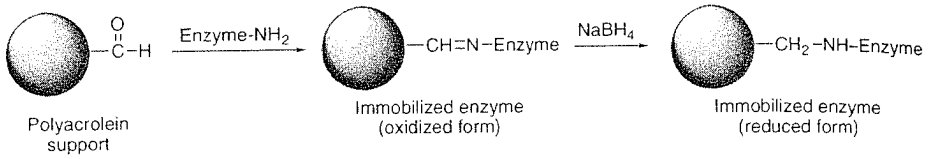


Fig. 5. Enzyme immobilization to unmodified polyacrolein via free amino groups, followed by reduction of the Schiff base with sodium borohydride.

1.5. Assaying the Properties of Immobilized Enzymes

There are three important properties of immobilized enzymes that are often evaluated: activity, enzyme loading, and stability. Prior to the measurement of these properties, the immobilized enzyme materials should be washed extensively to remove any unbound enzyme that may be entrapped in the pores of the particles or loosely bound through noncovalent interactions.

1.5.1. Activity Assay

There are two basic methods to measure activity—batch and continuous. In the batch method the immobilized enzyme is added to a flask or vial and the substrate solution is then added to initiate the reaction. At various time points, an aliquot of the mixture is removed and filtered (this is most easily done through a syringe filter) to remove any of the immobilized enzyme particles and to quench the reaction. This aliquot can then be analyzed using the appropriate analytical method, such as liquid chromatography, gas chromatography or spectrophototometry. If product continues to be produced in this aliquot after filtration, it is a good indication that there may be significant leaching of soluble enzyme off the support. This can occur if the support is not washed extensively enough after immobilization or if the binding is labile under the assay conditions. To get more accurate activity measurements the supports should be rewashed.

There are two basic methods for performing a continuous activity assay. In the packed-bed plug-flow tubular reactor (PFTR) method, the immobilized enzyme is packed into a column and substrate is pumped though the column and the substrate and/or product concentration is measured in the effluent. In the continuous stirred tank reactor, the solution and the immobilized enzyme are well mixed so there are minimal concentration gradients. To prevent the loss of immobilized enzyme out of the exit, a filter is added at the effluent or a tube is added at the exit that is long enough such that at the given flow rate gravity prevents the particles from leaving the reactor. Modeling a batch or continuous immobilized enzyme reactor can be found in many reactor engineering or bioprocess engineering textbooks.

1.5.2. Determining Protein Loading in Covalently Immobilized Enzyme Materials

It is often useful to have information on how much enzyme is bound to the support after an immobilization was performed. This information is needed when optimizing the immobilization conditions or when calculating the residual enzyme activity. Measurement can be done either indirectly or directly on the particles themselves.

In the indirect method, the initial amount of protein offered to the support is determined using any of the variety of protein assays available. After the immobilization is complete and the particles are washed, the same protein assay is done on the supernatant and wash solutions. The difference in the mass of enzyme offered and the amount in the immobilization supernatant and wash solutions will give the amount of enzyme bound to the support.

In the direct method, the amount of enzyme actually bound to the support is determined. A number of methods to determine this have been published. In one method, the bicinchoninic acid protein assay (often referred to as the BCA assay) is used *(45)*. In this assay, the immobilized enzyme is incubated in the BCA assay solution. The enzyme bound to the support reacts with the BCA solution in the same manner a soluble enzyme would, by reducing Cu^{2+} to Cu^{1+} in the presence of peptide bonds, which complex with the bicinchoninic acid to form a aqueous purple-colored solution. The absorbance of this solution will be proportional to the amount of enzyme immobilized. Bovine serum albumin (BSA) is often used as protein standards to quantify the amount of enzyme bound to the support.

Coomassie-based protein dyes have also been used to directly quantify the amount of enzyme bound to a support. In one method, the dye is allowed to bind to the enzyme attached to the support, after which the residual unbound dye is removed from the particles *(46)*. The bound dye is then eluted from the particles by adding sodium dodecylsulfate and sodium bicarbonate. The absorbance of this solution can then be read at 595 nm and the amount of protein bound to the support can be determined by comparing to a BSA standard curve.

In another method, the Bradford Protein Assay solution (a Coomassie-based protein assay) is used *(47)*. With no protein present, the Bradford solution is brown with a λ_{max} of 465 nm (with protein the color is blue with a λ_{max} of 595 nm). When the Bradford solution is mixed with the immobilized enzyme particles, some of the dye will bind to the protein on the beads (turning them blue) and the absorbance at 465 nm will decrease. This decrease at 465 nm can be correlated to the amount of bound protein by comparing to a standard curve of BSA or other suitable protein. The Bradford solution is acidic, so any enzyme that is immobilized through acid-labile links cannot be used with this method. Also, if the supernatant turns blue it is a good indication that significant leaching of the enzyme off the support is occurring.

Other methods also exist, including photometric, fluorometric, radiochemical, and ELISA-based methods. Elemental analysis on nitrogen or sulfur can also be used as long as the support material does not contain these elements. Amino acid analysis after acid hydrolysis can be used as well to determine protein content. All of these have differing sensitivity, work-up, and costs, all of which should be considered *(48)*.

1.5.3. Determining Stability of Covalently Immobilized Enzymes

Stability of immobilized enzymes can be measured in a number of ways. The simplest way is to pack the immobilized enzyme into a continuous reactor such as a column (plugged flow reactor) or a vessel with mixing (continuous stirred tank reactor). Substrate is then pumped through the reactor and the effluent is analyzed for product and/or substrate concentration. Depending on the stability of the enzyme, this is allowed to run for days or even months and the decrease in product concentration or the increase in outlet substrate concentration is monitored to determine the working lifetime of the immobilized enzyme. It is important to choose the proper amount of immobilized enzyme and flow rate such that less than 100% conversion is obtained. If 100% conversion is achieved, then it is unknown whether the entire immobilized enzyme takes part in the reaction. For example, if only half the enzyme present in the reactor is needed for 100% conversion under a given set of reaction conditions, significant inactivation (up to 50% of the enzyme) could occur with no observable change in conversion. The continuous reactor can also be run with various cosolvents or at various pHs or temperatures to determine the stability of the immobilized enzymes under these conditions.

The stability of immobilized enzymes can also be determined batchwise. In this technique, measured amounts of the immobilized enzyme are placed into separate vials along with the solvent of interest. The vials are allowed to incubate at a given temperature for a given amount of time. One or more vials are then sacrificed and the activity of the immobilized enzyme is measured (alternatively, the immobilized enzyme particles can be washed of substrate and product and returned to the initial incubation conditions). This is repeated over time to determine how the activity changes. Another batchwise method of measuring stability is to add the substrate solution to the immobilized enzyme and measure the reaction time course. The immobilized enzyme particles are then washed and this is repeated. The time it takes to reach the required conversion, the conversion at a set time, or the reaction time course can be plotted versus the number of recycles to give an indication of long-term stability/usability. Reactors capable of performing this automatically are commercially available *(36)*.

1.6. Industrial Uses of Covalently Immobilized Enzymes

A large number of enzymes are used in industry for the synthesis of a wide range of compounds. Most of these applications can be placed in either the food industry (both for human and animal consumption) or the pharmaceutical/fine chemical industry. Many of these applications involve immobilized enzymes with some of them covalently immobilized. A few examples of the latter will be discussed in **Subheadings 1.6.1., 1.6.2.,** and **1.6.3.**

1.6.1. High-Fructose Corn Syrup With Immobilized Glucose Isomerase

The largest use of immobilized enzymes is for the isomerization of glucose, from corn, to the much sweeter fructose. The resultant high-fructose corn syrup (HFCS) is used as a sweetener in a variety of foodstuffs, especially sweetened beverages and baked goods. Current US production of HFCS exceeds 9.3 million short tons (dry weight) per year (http://www.ers.usda.gov/briefing/sugar/ Data/Table27.xls). Glucose isomerase (also called xylose isomerase due the high affinity of the commercially available catalysts toward xylose) is used industrially to perform this reaction. Many of the commercial catalysts are immobilized whole cells; however, Miles Kali-Chemie (Germany) developed a glucose isomerase from *Streptomyces rubiginosus* covalently immobilized onto silanized ceramics and sold under the trade name Optisweet ***(49,50)***. Typically this reaction is run at 50–60°C to limit microbial contamination. The reactor is a continuous packed-bed reactor with a residence time of 0.17–0.33 h. The half-life of the enzyme is over 100 d; however, it is replaced after about 12.5% activity loss to maintain the necessary activity for the reactor ***(49)***.

1.6.2. Semi-Synthetic Penicillins with Immobilized Penicillin Amidase

Another major use of immobilized enzymes is in the synthesis of semi-synthetic penicillins. Worldwide production of these semi-synthetic β-lactam antibiotics is more than 20 thousand tons per year ***(49)***. The starting material for these compounds is 6-aminopenicillanic acid (6-APA). It is too expensive to make 6-APA synthetically or by fermentation. Instead, benzyl penicillin (also called penicillin G), which can be made relatively cheaply via fermentation, is hydrolyzed by penicillin amidase (also called penicillin acylase) into 6-APA and phenylacetic acid. This is accomplished industrially by using penicillin amidase from *Escherichia coli* or *Bacillus megaterium* immobilized onto Eupergit C. The reaction is usually carried out in a stirred-tank batch reactor. The immobilized enzyme can be reused nearly 1000 times before the half-life is reached. About 1700 kg of penicillin G is able to be hydrolyzed with 20 g of protein immobilized onto 300 g of dry polymer ***(51)***. Once the 6-APA is formed and purified, it is chemically acylated with various side groups to make the semisynthetic antibiotics such as

Fig. 6. Synthesis of L-aspartate from fumaric acid and ammonia catalyzed by L-aspartase.

ampicillin (D-phenylglycine side chain), amoxicillin (D-*p*-hydroxyphenylglycine side chain), and penicillin V (phenoxymethyl side chain). The preparation of a new immobilized penicillin G acylase yielding derivatives thermoestable and resistant to organic solvents is shown in **Chapter 17**.

1.6.3. L-Amino Acids with Immobilized L-Aspartase

L-amino acids are produced in large quantities for human consumption in the form of supplements, ingredients in artificial sweeteners, intermediates in the synthesis of pharmaceuticals, and as additives in animal feed. Covalently immobilized enzymes have been used to produce L-aspartase, a main ingredient in the artificial sweetener aspartame. This amino acid can be synthesized from two inexpensive starting materials, fumaric acid and ammonia (**Fig. 6**). The enzyme that catalyzes this reaction is L-aspartate-ammonia lyase, also called L-aspartase. This enzyme, from *E. coli,* has been covalently immobilized onto PVC-silica supports that have been coated with a polyamine and activated with glutaraldehyde. The process is relatively simple. The two starting materials are passed through a packed bed containing the immobilized enzyme. The effluent is acidified to pH 2.8 and chilled, causing the L-aspartate to precipitate, and it can then be recovered by filtration. Conversion, optical purity, and chemical purity of this reaction are all over 99%. The product concentration is 200 g/L with a space time yield of 3 kg/L·h. The reactor volume was 75 L, therefore producing more than 5 metric tons of product per day. The half-life of the immobilized enzyme was more than 6 mo ***(49,52)***.

1.7. Conclusions

Covalent immobilization of enzymes represents a robust method for the attachment of enzymes to insoluble supports. A variety of supports are available including synthetic and natural organic polymers and inorganic minerals, metal oxides, and glasses. The chemistry and functional groups used to bind enzymes to the supports can vary greatly and can be tailored depending on the specific application. In addition to the ease of enzyme recovery, stability of the immobilized enzyme is usually much higher than the soluble enzyme and thus can be

reused multiple times. Applications of covalently immobilized enzymes have been demonstrated from the lab scale to the industrial production of multiton quantities. Increasing numbers of enzyme are becoming commercially available in immobilized form, including lipases, proteases, nitrilases, amino acid dehydrogenases, oxidoreductases, and others *(53)*. This trend will continue as enzymes find more applications, particularly for chiral chemical production. This is a vibrant field that continues to evolve to this day.

2. Materials

2.1. Covalent Enzyme Immobilization

2.1.1. Covalent Attachment Onto Polyhydroxyl Supports

1. Polysaccharide support material, such as Sepharose 4B, Sephadex, agarose, cellulose, or dextran.
2. 2 *M* sodium carbonate.
3. 1 g/mL cyanogen bromide (CNBr) dissolved in DMF.

2.1.2. Covalent Attachment Onto Carboxylic Acid-bearing Supports

1. Carboxylic acid-bearing support such as Bio-Rex 70 Resin (BioRad, Hercules, CA), IRC50 (Supelco, St. Louis, MO), carboxymethylcellulose, acrylic acid copolymers, or succinylated glass beads.
2. 0.1 *M* sodium acetate buffer, pH 4.5.
3. 1-Cyclohexyl-3-(2-propyl)carbodiimide (EDC).
4. 0.1 *M* sodium phosphate buffer, pH 7.0.

2.1.3. Covalent Attachment Onto Amine-Bearing Supports

1. Amine-containing support, such as aminopropyl-glass, aminoethyl-cellulose, PEI-coated materials (e.g., silica or alumina) or other similar material.
2. 0.05 *M* phosphate buffer, pH 7.0.
3. 25% glutaraldehyde (GA).

2.1.4. Covalent Attachment to Reactive Polymer Supports

1. Eupergit C (Röhm Pharma Polymers, Piscataway, NJ).
2. 0.05 *M* phosphate buffer, pH 7.0.

2.2. Determining Protein Loading in Covalently Immobilized Enzyme Materials

2.2.1. Indirect Method

1. Immobilized enzyme.
2. Bradford reagent (Sigma Chemical Co., St. Louis, MO).
3. Bovine serum albumin (BSA) protein standards.
4. 0.05 *M* phosphate buffer, pH 7.0.

2.2.2. Direct Method (BCA)

1. Immobilized enzyme.
2. BCA protein assay kit (contains Reagent A and Reagent B).
3. BSA protein standard.

3. Methods

3.1. Covalent Enzyme Immobilization

3.1.1. Covalent Attachment Onto Polyhydroxyl Supports

1. Wash the support material with distilled water and remove residual water using suction filtration to form a packed cake.
2. Add 10 g of washed support material to a flask and add 2 *M* sodium carbonate until the total volume is about 1.2 times the settled bed volume (*see* **Note 1**).
3. Cool slurry to 0°C.
4. Add 1 mL of 1 g/mL of CNBr dissolved in DMF and mix vigorously for 2 min (*see* **Note 2**).
5. Wash the now-activated support material with at least 5 volumes of cold distilled water.
6. Add the activated support material to a solution of the enzyme in the appropriate buffer. Let incubate at 4°C for 12 to 24 h (*see* **Notes 3–5**).
7. Wash the immobilized enzyme material with the buffer used in **step 6.**

3.1.2. Covalent Attachment Onto Carboxylic Acid-Bearing Supports

1. Add 5 mL of the hydrated support to 15 mL of 0.1 *M* sodium acetate buffer, pH 4.5.
2. Add 200 mg EDC to the support slurry. Mix for 20 min at room temperature (*see* **Note 6** and **Note 7**).
3. Wash the beads with 500 mL of cold 0.1 *M* sodium phosphate buffer, pH 7.0.
4. Add the activated beads to 15 mL of the enzyme solution and gently agitate for 24–48 h at 4°C (*see* **Notes 8** and **9**).
5. Wash the immobilized enzyme beads with at least 500 mL of 0.1 *M* sodium phosphate buffer, pH 7.0.

3.1.3. Covalent Attachment Onto Amine-Bearing Supports

1. Suspend 10 g of the moist amine-bearing support in 100 mL of 0.05 *M* phosphate buffer, pH 7.0.
2. Add 10 mL of 25% glutaraldehyde (GA) and mix well for 1–2 h (*see* **Notes 10** and **11**).
3. Wash and filter the GA-activated support material with 500 mL of the above buffer at least five times to remove any residual GA (*see* **Notes 12** and **13**).
4. Suspend 10 g of the moist GA-activated support in 30 mL of the enzyme solution in the above buffer and mix well at room temperature or 4°C depending on enzyme stability (*see* **Note 14**).
5. After 12–24 h filter the immobilized enzyme material and wash well with the above buffer (*see* **Note 15**).

3.1.4. Covalent Attachment to Reactive Polymer Supports

1. Add 1.0 g of dry Eupergit C to 6.0 mL of the enzyme solution in 0.05 *M* phosphate buffer, pH 7.0 (*see* **Notes 16–19**).
2. Gently mix the suspension at room temperature or 4°C depending on the stability of the enzyme.
3. After 24–48 h wash the immobilized enzyme with 50 mL of the buffer in **step 1** followed by suction filtration. Repeat the washing three to five times (*see* **Note 20**).

3.2. Determining Protein Loading in Covalently Immobilized Enzyme Materials

3.2.1. Indirect Method

1. Before adding the enzyme solution to the support for immobilization, record the volume of the enzyme solution and the dry weight of the support.
2. Determine the protein concentration in the enzyme solution (prior to adding it to the support material) using the Bradford protein assay method. This is done by adding 0.1 mL of the enzyme solution to 0.9 mL of Bradford reagent and mixing. OD_{595} is recorded after it has stabilized (usually 5–10 min). Protein concentration is calculated by comparing to a standard curve using a suitable protein standard such as BSA. The linear range for this method is approximately 0–0.5 mg/mL protein, therefore, if necessary, the enzyme solution should be diluted in the phosphate buffer (*see* **Note 21**). After the immobilization is complete, pour off the supernatant into a separate container.
3. Wash the immobilized enzyme as necessary and add the washings to the supernatant in **step 2.**
4. Record the combined volume of the supernatant and washings (from **step 3**) and measure the protein concentration as described in **step 2** (*see* **Notes 22–24**).
5. Use the following equation to calculate the amount of protein bound:

$$\frac{\text{mg enzyme bound}}{\text{g dry wt of support}} = \frac{\left(\text{enzyme conc. in initial solution (mg/mL)} \times \text{volume of initial solution (mL)}\right) - \left(\text{enzyme conc. in final solution (mg/mL)} \times \text{volume of final solution (mL)}\right)}{\text{g dry weight of support}}$$

3.2.2. Direct Method

1. Make up 20 mL of the BCA working solution by mixing 19.6 mL of reagent A with 0.4 mL of reagent B.
2. Make up 1.0 mL BSA stock solution of 25.2 mg/mL.
3. In 3–5 mL (total volume) capped tubes make up the following solutions (add the working solution last) (*see* **Notes 25** and **26**):

Sample	BSA standard, 25.2 mg/mL (μL)	Water (μL)	Final BSA conc (mg/mL)	Immobilized enzyme (mg dry)	Working solution (mL)
Std-1	0	100	0	0	2.0
Std-2	8.3	91.7	0.1	0	2.0
Std-3	25	75	0.3	0	2.0
Std-4	50	50	0.6	0	2.0
Std-5	75	25	0.9	0	2.0
Std-6	100	0	1.2	0	2.0
Immobilized enzyme	0	100	N/A	10–20	2.0

4. Shake the standards solutions and immobilized enzyme suspensions well at 37°C for 30 min, then cool the tubes to at or below room temperature.
5. Remove particles by filtration or centrifugation and measure OD_{562} of the supernatant (*see* **Notes 27–29**).
6. Construct a calibration curve using the BSA standards, using the 0 mg/mL BSA sample as a blank (*see* **Notes 30–32**).
7. Based on the calibration curve calculate the protein concentration in the immobilized enzyme samples.
8. Use the following equation to determine the enzyme loading:

$$\frac{\text{mg enzyme bound}}{\text{g dry weight}} = \frac{\text{concentration of protein in immob. enzyme sample (mg/mL)} \times 2.1\ \text{mL}}{\text{amount of immob. enzyme used in assay (g dry weight)}}$$

4. Notes

1. A procedure similar to this can be performed where, instead of using concentrated buffer, the pH is maintained at 11.0 by titrating with 2 or 4 *N* NaOH. This method often results in the doubling of the binding capacity compared to the buffer method.
2. CNBr is highly toxic and proper safety precautions should be employed when handling it.
3. Although the activated support materials should be used soon after activation, it can be stored at –20°C under airtight conditions with a loss of 10% or less per month of its original binding capacity.
4. Ideally the enzyme should be in a buffer at an alkaline pH to reduce the amount of protonated amino groups. However, it is more important to have the enzyme at a pH it is most stable, so this should be chosen if the enzyme is not stable at high pH.
5. The binding of protein to the support can be monitored by performing protein assays on the supernatant (e.g., Bradford or BCA total protein assay) and comparing to the initial protein concentration. For some enzymes, incubations times longer than 24 h may be necessary to achieve maximal enzyme binding.

6. 1-Cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide metho-*p*-toluene-sulfonate (CMC) may also be used to activate carboxylic acid-containing supports.
7. The EDC concentration is about 100-fold molar excess to the carboxylate groups on the support. The activated support should be washed well before adding enzyme.
8. EDC concentration, activation time, coupling time, coupling pH, and wash conditions (i.e., washing with NaCl and/or urea to remove any unbound enzyme) should all be optimized for a given support-enzyme system.
9. An alternative method is to add the support, EDC, and enzyme all at once such that support activation and enzyme immobilization occurs simultaneously. In this case, a 1:1 molar ratio of EDC to support carboxyl groups should be used. Allow this to react in 0.05 *M* sodium phosphate buffer, pH 7.0, at 4°C for 1 h before washing. Longer times may be necessary depending on the enzyme and support. The EDC may also react with the carboxyl groups on the enzyme causing inactivation. If this occurs, the other method should be used.
10. A higher concentration of GA can be used and this may increase the binding capacity of the support material. However, since GA is often detrimental to the enzyme, more extensive washing should be performed.
11. If PEI coated materials are used, they will turn to a pinkish color after activation with GA.
12. A saturated solution of 2,4-dinitrophenylhydrazine in 0.2 *N* HCl can be used to detect residual GA in the washing solutions. Add approx 0.2 mL of the solution used to wash the GA-activated support to 0.5 mL of saturated 2,4-dinitrophenylhydrazine solution. The formation of a yellow precipitate indicates the presence of residual GA and the support material should be further washed.
13. The GA-activated support can be stored in a moist form at 4°C for at least 1 yr without significant loss in binding capacity.
14. Shaking as opposed to magnetic stirring should be used if the support is friable.
15. Enzyme loading in this type of immobilization can be as high as 30% dry weight and higher.
16. The dry Eupergit C will absorb about 3 times its weight in water (1 g dry will have a mass of about 4 g when wet).
17. The Eupergit should be kept dry until use as the epoxy groups can hydrolyze.
18. Approximately 5–10% enzyme loading on a dry basis is typical, however this is dependent on the enzyme and higher or lower loading levels are possible.
19. The ionic strength and the pH of the enzyme solution can significantly affect the loading amount and the residual activity. Often a high ionic strength (1 *M* sodium chloride) gives better binding, but this is dependent on the enzyme and a range of ionic strengths and pH values should be evaluated.
20. Post-treatment of the beads after immobilization to quench the remaining epoxy groups can effect the immobilized enzyme activity. *See* **Subheading 1.4.4.** for details on this.
21. Other protein assays may be used besides the Bradford assay, i.e., BCA, Lowry, absorbance at 280 nm.

22. If any enzyme precipitates during the immobilization, this could overestimate the amount of enzyme bound.
23. If the amount of enzyme bound to the support is very small, the inherent variability in the protein assay may not give accurate protein loading results.
24. If the wash solution contains compounds that interfere with the protein assay, unreliable data may result.
25. This method is a general method and can be modified as necessary depending the enzyme loading. The method above assumes a 1–10% enzyme loading based on dry weight.
26. The BCA protein assay has a working range of 0.02–2 mg/mL protein.
27. For low loading or small sample sizes the "enhanced protocol" can be used (incubation at 60°C for 30 min).
28. This should be done as soon as possible after cooling as the standards will continue to react with the BCA reagent while the immobilized enzyme will not if removed or centrifuged.
29. If the absorbance of the immobilized enzyme samples falls outside the range of the calibration curve, then the procedure should be repeated with a change in either the standards or immobilized enzyme concentration.
30. For more accurate results, the standards and the immobilized enzyme samples should be done in at least duplicate.
31. The support without any enzyme bound should also be tested to see if the BCA shows a response toward it. If it does, this should be taken into account and subtracted from the results of the immobilized enzyme supports.
32. The standards should be used every time this procedure is performed as the assay is highly dependent on the temperature and time of incubation, which may be difficult to replicate every time.

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