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Lipid Sorption of Contact Lenses Using Radiolabeling Techniques

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Abstract

Lipid sorption on silicone hydrogel contact lenses is often measured using chromatographic methods, but there are controversies regarding the accuracies of these methods. Radiolabels give precise and accurate results, but require infrastructure and are still subject to efficient removal from the lens. This study compares 3 methods of quantitating phospholipid and cholesterol sorption to silicone hydrogel lenses using radiolabeled lipids that were sorbed to lenses from an artificial tear solution. A triple extraction technique using n-propanol gives the most quantitative results. Comparison of sorption on silicone hydrogels shows that balafilcon and senofilcon lenses sorb similar amounts, but more than lotrafilcon lenses.

Introduction

Lipid and protein adsorption to contact lenses is a known complication of contact lens wear that can lead to user discomfort and decreased visual acuity.[1] While traditional hydrogels are more likely to adsorb protein, silicone hydrogels (SiHy) are more apt to adsorb lipid. Lipid adsorption is commonly measured by chromatography methods as this allows for simultaneous measurement of multiple lipid species from complicated lipid/protein solutions, as well as after clinical use. Gas chromatography/mass spectrometry (GC/MS) is more

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sensitive than liquid chromatography (HPLC) for the identification and determination of lipids adsorbed on SH lenses but involves more cumbersome sample preparation.

Chromatography techniques, while useful, are not without controversy. Jones et al[2] reported high levels of lipid adsorption to balafilcon lenses. Maziarz et al[3] reported much lower levels and also demonstrated that small differences in HPLC methods can result in substantial differences in results.

Compared with chromatographic methods, radiolabels are definitively accurate but less useful for studying simultaneous sorption of multiple lipid species because each species requires a different radioisotope (or a different experiment for each different species). Since Prager and Quintana[4] measured uptake of 3H-cholesteryl oleate and 14C-dioleoyl phosphatidylcholine from a multi-component artificial tear fluid (ATF) to traditional hydrogels in 1997, little has been published on the use of radiolabels to study protein and lipid sorption to silicone hydrogel contact lenses. While chromatographic methods have become the norm, few such studies have also utilized independent techniques such as radiochemistry to validate extraction procedures or chromatography results. As Butovich cautioned, "Often, a researcher is tempted to use a method that is either cheaper, or simpler, or more available at the moment, instead of using a technique which provides better results, albeit at the expense of time, convenience, or higher costs."[5] Therefore, in this study radiolabeled lipids were used to study lipid adsorption to SiHy lenses and to compare the results with published literature based upon chromatographic analysis.

Materials and Methods

Materials. Cholesterol (CH) labeled with 14C in ethanol was purchased from Perkin Elmer. Dipalmitoylphosphatidylcholine (DPPC) labeled with 3H in ethanol was purchased from American Radiolabeled Chemicals, Inc. Lysozyme, lactoferrin, and albumin were purchased from USB Corporation. All additional materials were purchased from Sigma-Aldrich.

Four lens types were tested: Acuvue Oasys (senofilcon A), Pure Vision 2 (balafilcon A), Air Optix (lotrafilcon), and SofLens (polymacon). The lenses were in commercial blister packs obtained from distributors. Acuvue Oasys and Pure Vision lenses were tested using all three methods of extraction. After it became apparent that n-propanol extraction was most efficient, the SofLens and Air Optix lenses were tested using only n-propanol extraction.

Preparation of Artificial Tear Solution

Artificial Tear Solution. Borate buffered saline (BBS) was prepared by combining 5g boric acid, 0.6g sodium borate and 2g sodium chloride in 500 mL of distilled deionized water (DDH2O). The solution pH was 7.3. The artificial tear solution (TS) was prepared by adding to a 100 mL volumetric flask 0.54mL of 0.03 w/v% 14C-cholesterol in ethanol, 0.108mL of 0.00025 w/v% 3H -DPPC in ethanol, 0.19mL of 16mg/mL CH in chloroform (chlf), 0.2mL of 16mg/mL DPPC in chlf, 0.2mL of 40mg/mL methyl-ß-cyclodextrin in chlf, 0.2mL of 33 mg/mL cholesteryl linoleate in chlf, 0.2mL of 2mg/mL oleic acid in chlf, 0.2mL of 44mg/mL methyl oleate in chlf, and 0.2mL of 5mg/mL triolein in chlf. The mixture was dried under a nitrogen stream followed by

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Nanomechanical Characterization of Hydrogel Contact Lenses

By Amanda Simpson and Richard Nay, Hysitron, Inc.

Introduction

Nanomechanical testing has become a growing technique for characterizing hydrogel materials. Hydrogels are networks of polymer chains that are hydrophilic and very similar to natural tissues due to their high water content. Some common applications for hydrogels include tissue scaffolds, drug delivery systems, and contact lenses.

Since hydrogels are gaining popularity, especially in the medical field, it is important to understand their mechanical properties. Nanomechanical testing is a valuable option for testing these types of materials because little to no sample preparation is required, only a very small testing region is needed which allows samples to be tested in their natural state and which provides highly localized mechanical property measurements, it is a virtually non-destructive testing technique, and it has the flexibility to test materials in the fully hydrated state.

Experimental

Nanomechanical testing was performed on three types of commercially available hydrogel daily wear contact lenses using a TI 950 TriboIndenter® nanomechanical testing instrument equipped with nanoDMA® III and an XZ-500 Extended Displacement stage (Hysitron, Inc., Minneapolis, MN). The instrument was used to perform dynamic and quasi-static nanomechanical tests on commercial samples we refer to as Type 1, Type 2, and Type 3 lenses. The Type 2 and Type 3 lenses were from the same manufacturer.

First, nanoDMA tests were performed on each type of lens to characterize the time dependent properties of the material. The nanoDMA technique is comparable to conventional compressive dynamic mechanical analysis testing techniques, but on a much smaller spatial scale. Figure 1 compares storage modulus results for nano and macro DMA testing on the same set of silicone samples with varying amounts of crosslink density. The results from both techniques are in good agreement.

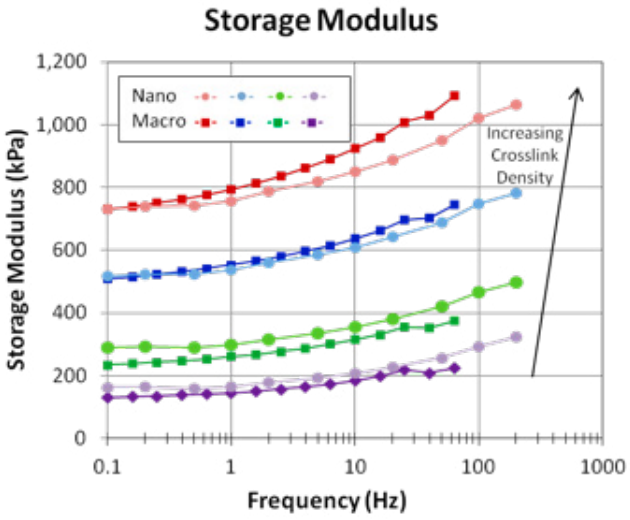

Figure 1. Macro versus nanoDMA comparison on silicone with increasing amounts of crosslink density.

Since silicone and hydrogels have similar magnitudes of mechanical properties the nanoDMA technique was used to test contact lenses, no special sample geometry was required and the lenses were tested "as is" using a custom dome-shaped sample mount. For testing, a 100 µm diameter diamond cylindrical flat punch probe was pushed into the surface of the sample using a constant normal quasi-static force of approximately 1,000 µN, while at the same time a dynamic oscillating force was applied to reach displacement amplitudes of approximately 40 nm. The frequency was ramped from 0.1 to 50 Hz in 18 individual steps. Storage modulus (E'), loss modulus (E"), and tan delta (tan δ) values of each sample were then calculated from the following equations, where ks is the stiffness of the sample-probe contact, A is the contact area, ω is the angular oscillation frequency, and Cs is the damping attributed to the sample-probe contact:

a) $E' = \frac{k_s\sqrt{\pi}}{2\sqrt{A}}$

b) $E'' = (\omega C_s \sqrt{\pi})/(2\sqrt{A})$

c) $\tan\delta = E''/(E')$

Figure 2 displays storage modulus, loss modulus, and tan delta versus frequency for the series of nanoDMA tests performed on the Type 1, Type 2, and Type 3 lenses. For each sample the storage modulus increases with increasing frequency, as expected with this type of material. The tan delta and loss modulus results show trends that clearly differentiated the properties of the hydrogel materials used by the two manufacturers. The hydrogel from the first manufacturer (Type 1) exhibited a tan delta minimum at approximately 10 Hz. The hydrogels from the second manufacturer (Type 2 and Type 3) had tan delta minima at approximately

Nanomechanical Characterization Continued on Page 4

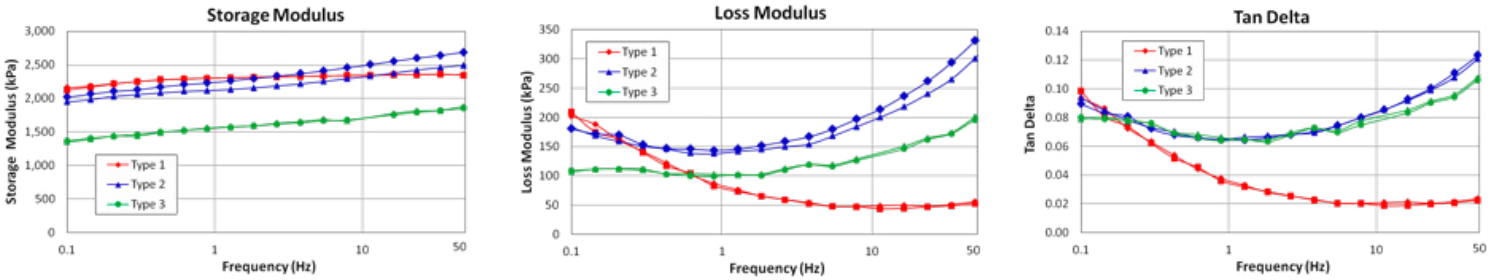

Figure 2. Storage modulus, loss modulus, and tan delta versus frequency from two separate nanoDMA tests performed on each type of lens. The Type 2 and Type 3 lenses were produced by the same manufacturer. First and second tests were in good agreement for each lens.

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1 Hz. The tan delta values versus frequency from these two samples were remarkably similar even though their storage modulus values noticeably differed.

Nanoindentation

The storage modulus results from the nanoDMA testing yielded mechanical properties as a function of frequency. To complement this data, quasi-static nanoindentation tests were performed to characterize the materials at a variety of depths. Each lens was approximately 250 µm thick. A modulus versus depth study was performed using a diamond conical probe with a 20 µm radius of curvature with a 90° included angle and the XZ-500 Extended Displacement stage which allowed for testing at depths ranging from ~1 nm to 500 µm. The conical probe was selected for nanoindentation because the flat punch probe would not be in full contact with the curved surface of the sample at shallow depths. Nanoindentation tests were performed on the Type 1 and Type 2 lenses (which exhibited similar storage moduli) to contact depths from ~2 to 30 µm. For each test the probe was displaced into the surface to a specified depth, held for a period of 10 seconds to allow for sample creep to subside, and then withdrawn. Reduced modulus (Er) and hardness (H) were calculated from the resulting force versus displacement curves from the following equations, where S is the initial stiffness of the unloading curve, A is the contact area, and Pmax is the maximum force applied:

$$(a)\ E_r = (S\sqrt{\pi})/(2\sqrt{A}) \qquad b)\ H = P_{max}/A$$

Figure 3 shows force versus displacement curves from the series of indents performed on each lens. Figure 4 compares the hardness and reduced modulus versus contact depth results for each lens. As contact depth is increased the hardness and reduced modulus of the samples also increase. For both samples there seems to be consistent mechanical properties at contact depths less than 5 µm, and as contact depth increases the reduced modulus also increases. Further testing could include nanoDMA tests being performed at various contact depths to fully characterize the materials dynamic and depth characteristics.

Conclusion

Nanomechanical testing was successfully used to characterize the mechanical properties of three separate hydrogel contact lens samples. Understanding these properties is important as these types of materials grow more popular in the medical field. The nanoDMA testing technique was a valuable and non-destructive tool used to characterize the viscoelastic properties as a function of frequency. This testing along with nanoindentation to select proper depth ranges would be especially useful in research and development of new materials, quality control of existing materials, and new product validation.

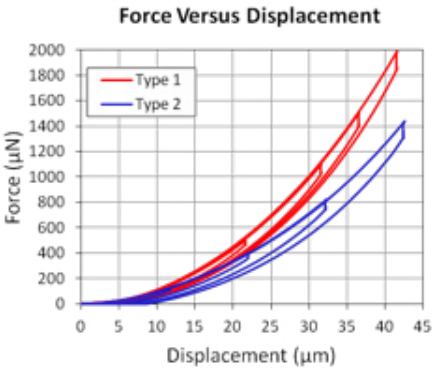

Figure 3. Force versus displacement curves from indents performed on the Type 1 and Type 2 lenses at various depths.

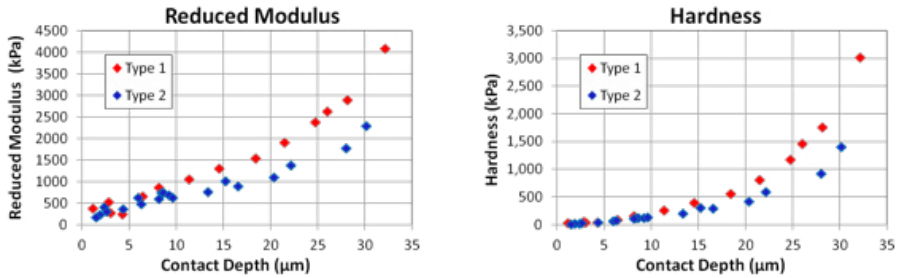

Figure 4. Reduced modulus and hardness versus contact depth from indents performed on the Type 1 and Type 2 lenses which yielded similar storage moduli from nanoDMA testing. For both samples there seems to be consistent mechanical properties at contact depths less than 5 µm.

One Size Does Not Fit All: Drafting Patent Applications and Sufficiency of Disclosure

By Colin Fairman, JD, PhD, Intellectual Property and Legal Editor

As discussed in the Winter 2011 issue of *SurFacts*, the passage of the America Invents Act (AIA) changed the way that inventorship is determined—the U.S. went from a "first to invent" system to a "first to file" system. This change, however, prompts the question regarding the new system's effect on the other criteria for obtaining a patent.

The Patent Office still requires novelty and non-obviousness. In other words, the claims must still be free from prior art; but what about the rest of the invention application?

Most of the criteria for obtaining a patent, unrelated to the actual prior art in the field of your invention, are governed by 35 U.S.C. § 112 which provides the requirements for the Specification. There are six paragraphs to § 112, but in this column I would like to focus on paragraph one, which provides:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

In short, paragraph one requires three things from the specification: (1) a written description; (2) enablement; and (3) the best mode for carrying out the invention. Now, some of you may remember that one of the modifications brought to patent law by the AIA was that "best mode" will no longer be a basis for challenging a patent. The patent office has told us that although "best mode" will still be a requirement for obtaining a patent, the Examiners will no longer question its absence (or presence). However, that still leaves two factors: (1) written description; and (2) enablement. What are these requirements and what do they mean? Moreover, does their meaning change with the subject matter of the invention?

Written Description

[T]o satisfy the written description requirement, an applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention, and that the invention, in that context, is whatever is now claimed. *The test for sufficiency of support in a parent application is whether the disclosure of the application relied upon "reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter."* *Ralston Purina Co. v. Far-Mar-Co.*, Inc., 772 F.2d 1570, 1575, 227 USPQ 177, 179 (Fed. Cir. 1985) (quoting *In re Kaslow*, 707 F.2d 1366, 1375, 217 USPQ 1089, 1096 (Fed. Cir. 1983)). MPEP 2163.02. (Emphasis added).

Now, how is the written description different from enablement?

Enablement

"[T]o be enabling, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without 'undue experimentation.'" In re Wright, 999 F.2d 1557, 1561 (Fed. Cir. 1993).

As you might guess, there has been considerable effort put forth by various patent law scholars to determine just what the difference between enablement and written description is, and how you can tell you have enablement. In my view, if you have a comprehensive written description, you should have your claims enabled. Sometimes, however, you have your claims enabled, but not to reflect the breadth of the written description. This is where the difference in technologies comes in.

In general, I have expertise in three fields and I dabble in a few more. Specifically, the main part of my practice is directed to mechanical devices, chemistry, pharmacology, molecular biology, and biotech (a very broad category depending on your definition, but here I use it to mean technical devices used in biology, chemistry and pharmacology [including drug delivery devices]). In addition, I have a small portfolio of laser applications and polymer/nano devices. I also do quite a bit of work on purely mechanical inventions. However, I do not practice in the areas of electronics and computers. So, while my experience holds for these fields as well, it is not firsthand.

Let's take the simplest example: a purely mechanical device. If you have invented a new widget, the widget may be novel because you have developed a two-barreled shaft to hold the widget, with the shafting having an inboard spring that keeps the widget from slipping on the sprocket. Now in the case of the new widget, a clear figure and figure legend showing the elements of the sprocket may be sufficient to provide all the written description and enablement you need.

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After all, a figure certainly conveys how the parts of the widget fit together and that would show that you—the inventor—were in possession of that spring on that two-barreled shaft.

Let's talk about chemistry. We may have invented a new thermoplastic elastomer or a new method for making that compound (if it is a new compound, the method must be new). In this case, we need to tell those of skill in the art information such as: what our starting reactants are; what the purity of the reactants is; which reactant is added first; which reactant is added second; and what the temperature, pressure, and atmosphere are. These are only some of the variables that might affect a chemical reaction. In the broadest sense, the written description of new thermoplastic elastomer (or the teaching of how to make it) will tell you that, according to your new method, an organic base is added to a buffer, at room temperature, a plastic elastomer is added, and the entire mixture is shaken at elevated temperature for a time and then cooled.

This description provides a very broad disclosure with a very broad genera of reactants. But, is it enabled?

In the case of the thermoplastic elastomer, we need to show those of skill in the art how to make it. This means we need examples. I ask my clients to give me as many examples as they can. Examples are invaluable because while they are quite easy to include in a specification, nothing goes further in showing a patent examiner that you know what you are talking about than actual data from experiments that you have performed.

Now, in the case of the thermoplastic, a number of variables need to be enabled to get broad coverage for the invention. In this case, "organic base," how many are there? Can they all be used? Buffer? What pH? Which thermoplastic do we use? The more species of each of these genera there are examples of, the greater the applicant's ability to show the patent examiner that the applicant knew that the universality of his or her method could encompass a very large genera of components. However, if only one example is provided showing only one species of each genera in a single set of reaction conditions, the applicant has nothing to fall back on when the Examiner tells him he can only have claims directed to the very narrowest of species/reaction conditions disclosed in the single example.

Next let's take pharmacology. For this technology, there is a big component of the chemistry method discussed above. However, the further part of this technology is showing that the compound worked for the claimed indication. In the context of pharmaceuticals, the Patent Office does not request nor require information regarding the safety and efficacy of the pharmaceutical compound. In fact, the Patent Office and the FDA recognize each other's area of jurisdiction, but that patent applicant does need to show the effect claim is related to the composition.

Now let's take another example—a new anti-obesity drug. After we tell those of skill in the art how to make the compound, we have to show that it works. Generally, the Patent Office will accept both in vitro and in vivo studies at face value. With the case of our new anti-obesity drug, we can show that cultured adipocytes cease to divide upon addition of the drug to the culture medium. Maybe tests show that the adipocytes die after two days. Maybe the data show that the adipocytes fail to take up glycerol from the media as they did prior to administration. Administration of the drug at various doses provides a graph showing there is a linear effect. These are all good data that enable our claim over a range of doses in inhibiting obesity by (claim 1) stopping the uptake of fats by adipocytes; killing adipocytes (next independent claim); stopping the division of adipocytes.

Now, suppose we have in vivo data taken using mice as the model. Mice given the drug lose weight. Mice given the drug cannot gain weight. Mice given the drug lose fat, but not muscle mass. Now we have enabled claims directed to: (1) a method of providing weight loss; (2) a method of inhibiting weight gain; and a method of decreasing the size of the fatty component relative to the muscle component of a patient in need. These are all nice claims that are enabled by the examples provided in the specification.

Next let's take molecular biology. Imagine that you have engineered a microorganism to produce and

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secrete diesel fuel and that these microorganisms are maintained on a standard diet of media. (Not so farfetched an idea—see here). To reduce this invention to practice (e.g., make it work), it may be necessary to identify a metabolic pathway that utilizes the required nutrients (here, assume it is glucose), provides some important byproduct, and inhibits a normal metabolic step to divert the normal glucose metabolism and insert a gene for an alcohol reductase to divert the normal metabolism from the production of simple sugars to a fatty-acid methyl ester (FAME), otherwise known as biodiesel.

In this scenario, one would have to provide a written description of how the purported production of FAME could be achieved using the biological mechanisms of E. coli (as an example of many microorganisms that could be used). In providing an adequate written description, one would have to discuss how to make genetic knock-ins, genetic knock-outs, and strategies for molecular biology such as whether one really needs a knock-in, or if instead, you could put the gene in a replicating plasmid with a constitutive promoter and/or an inducible promoter. One may need to discuss the benefits of retroviral plasmids in order to get a genomic insert. One would have to identify what percent of production should be considered success and how that is measured.

Then the inventor would have to enable claims directed to microorganisms' (not just E. coli) use of various endogenous pathways. The inventor would also have to enable claims regarding methods to disrupt a specific gene (in the genomic complement so it is not replicated), methods to insert a gene (or genes) into the genome (mutation of a native gene/insert of a foreign gene) use of promoters (e.g. inserting the gene behind a native promoter, inserting the gene with a foreign promoter, use of a constitutive promoter, use of an inducible promoter.

As a reflection of the difference in the complexity of the technology, consider that a simple mechanical patent application may be from 3 to 10 pages long. A simple chemical application may be up to 20 pages long. An application directed to a pharmaceutical may be 50 to 100 pages long. Generally, applications dealing with molecular biology would not be unusual at over 100 pages.

So, what is the difference between the written description requirement and enablement? In short, they are different sides of the same coin. The written description shows that you have made the idea concrete in as broad and complete disclosure to illustrate that the inventor is in full possession of the invention with all of its nuances. The enablement requirement is met when you show the world how to do it. Thus, enablement is directed to giving the inventor the broadest claims possible to protect the invention in its broadest context. With the example of making diesel fuel, this means we can show that other types of microorganisms can be used besides E. coli. Showing that these organisms share the same basic pathways, showing that one or many pathways can be mutagenized to alter metabolism and identifying the breadth of culture conditions at which the process can be carried out.

Generally, as a rule of thumb in the U.S., showing enablement of an invention with at least three different species provides some standing before the Patent Office to argue that the inventor deserves to claim a genus (all microorganisms/all organic bases/ all sugars). However, the readers should appreciate that the U.S. is comparatively lenient in this respect. Many patent regimes will only allow claims directed to those particular species/indications for use/conditions of reaction that are actually shown in a specification. Therefore, if you are considering filing a patent application in Europe and China as well as the U.S., it is advisable to do the experiments, describe your invention, and enable a broad set of claims. Remember: When it comes to patents, one size does not fit all regarding the sufficiency of the disclosure of your invention.

Surface Science Calendar of Events

Surface Characterization of Biomaterials - 2012 Annual NESAC/BIO Workshop
August 13-15, 2012
Seattle, WA
http://www.nb.uw.edu/home/workshop/

3rd TERMIS World Congress 2012
September 5-8, 2012
Vienna, Austria
http://www.wc2012-vienna.org/

Innovations in Biomedical Materials 2012
September 10-13, 2012
Raleigh, NC
http://ceramics.org/meetings/innovations-in-biomedical-materials-2012

BIOINTERFACE 2012

BioInterface 2012
October 23-25, 2012
Dublin, Ireland
http://www.BioInterface2012Ireland.com

AIChE 2012 Annual Meeting
October 28-November 2, 2012
Pittsburgh, PA
http://www.aiche.org/Conferences/AnnualMeeting/index.aspx

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several hours of vacuum drying. The lipids were rehydrated by stirring in ~50mL BBS for 8 hours before adding 2.68mg lysozyme, 3.58mg lactoferrin, 8.7mg albumin, and 10mg mucin and stirring for an additional 8 hours. Additional BBS was then added to the 100mL mark. 100µl standards were pipetted from the tear solution to vials containing 10mL of scintillation fluid and 10mL of scintillation fluid with 3mL of n-propanol.

Sorption to Lenses. 2mL of radiolabeled TS were pipetted into 5mL glass vials. Lenses were removed from their blister packs, rinsed in BBS and gently blotted on Kim Wipes® before being submerged in the TS in the vials. The lenses were placed in an incubator at 37°C on an orbital shaker for 16 hours. Lenses were then removed from the vials and rinsed with double distilled water before being processed to quantitate the sorbed lipids.

Direct Counting. Lenses were placed directly in 10 mL scintillation fluid in glass scintillation vials. The vials were counted immediately, and then at 2 hours, 4 days, and 7 days.

70/30 Chloroform/Methanol Extraction. Lenses were extracted by submersion in 2mL of a 70/30 vol% mixture of chlf/MeOH in a scintillation vial for 2 hours on an orbital shaker at 37°C. Then the lenses were rinsed into the same vial with additional extraction fluid before being removed. The solvent in the vials was evaporated in an incubator at 62°C until dried. The dried vials were then filled with 3 mL n-propanol and placed on an orbital shaker in an incubator at 37°C for 12 hours. Finally, 10 mL scintillation fluid was added to each vial before being counted.

n-Propanol Extraction. Each lens was placed in a scintillation vial containing 2 mL n-propanol on a shaker for 1 hour at 37°C. Lenses were then rinsed with 1 mL n-propanol into the same vial before being transferred to a second extraction vial containing another 2 mL of n-propanol. After 1 hour, it was rinsed the same way. Finally a third 1-hour extraction and rinse were done. Following the extractions, the lenses removed from the final vial were transferred to a vial filled with 10 mL SF to measure any residual radioactivity.

Counting. All vials were counted using a LS 6500 scintillation counter (Beckman Coulter) twice using a program that counted both the amounts of 14C and 3H. Blank vials containing only scintillation fluid with and without n-propanol were counted as background and used in the calculations.

Results and Discussion

In this study, radiolabeled DPPC and CH, which represent both hydrophilic and hydrophobic lipids in the tear films, were used to evaluate the sorption to SiHy contact lenses and to establish a quantitative analytical procedure for SiHy lenses. Previous quantitation of lipid sorption to SiHy contact lenses was done using chromatographic methods, which have some challenges in quantitation. The use of radiolabeled lipids requires institutional infrastructure and cumbersome safety procedures, but allows direct and exact quantitation of labeled lipids and provides tracking methods to determine the extraction efficiency from the contact lens.

Many sources of analytical error can be reduced by using radiolabeling techniques. For example, in this study we adjusted the amount of labeled lipid such that we used the same pipet and the same volume setting (0.100 mL) on the pipet to collect sample standards from each batch of TS solution and to collect the samples in the various techniques. Because the counts of the sample are ratioed to the counts of the standards (after subtracting background counts in each) any inaccuracy in pipet volume is nullified, and only the imprecision of repeated pipettings contributes to the overall error in the measurements. Also with radioactive techniques, the counting error decreases proportionally with the number of counts obtained. The experiments

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were designed such that in all the scintillation measurements, the counts were more than 10,000 cpm, which represents an accuracy counting error of less than 1%. The half-lives of these isotopes (12.32 yr for 3H and 5,730 yr for 14C) are sufficiently long that corrections for decay over the timecourse of these experiments would not be needed. However, the standards and the samples decay at the same rate, so correction for half-life decay is not needed, as long as the standards are counted at the same time as the samples (which they were). These and other considerations point to the accuracy in using radiolabeling techniques for quantitation of sorbed lipids on contact lenses.

Three different analytical methods were compared to evaluate their accuracy and precision in quantitating the amount of sorbed lipid.

Direct Counting

In the direct counting method, the lens was placed directly in the scintillation fluid (SF), and the counts per minute (cpm) used to quantitate the sorbed lipid. It was observed that the SiHy lenses (AO and PV) swelled 10 to 20% in diameter in the SF. After 16 hours of incubation in TS, the lenses were placed in a vial of SF and counted immediately. They were also counted at later times. The cpm associated with a vial increased with time and reach a plateau after about 4 days of incubation, as shown in Figure 1. The values of sorbed lipids are also much less than calculated from the other techniques (see Figs. 2 and 3).

Although direct counting would be most convenient, this measurement is fraught with problems as indicated by these data. First we note that the beta particle emission of 3H and 14C are fairly weak, and are absorbed by contact lens material as well as the scintillation fluid. In water, 3H beta emissions travel an average of about 0.42 µm before they are absorbed, and the more energetic 14C beta emissions travel about 19 µm before capture. Capture by SiHy materials has not been reported, but it is probably about the same order of magnitude. The majority of the emissions from lipids that absorb deeper than these lengths probably will be captured by the SiHy material before they can be captured by the SF. Only half of the emission from lipids that adsorb on the surface of the lenses are captured and counted by the SF. The other half of the emissions, which are emitted into lenses that are on the order of 100 µm thick, are probably never captured by scintillation fluid unless the SF has absorbed into the lens.

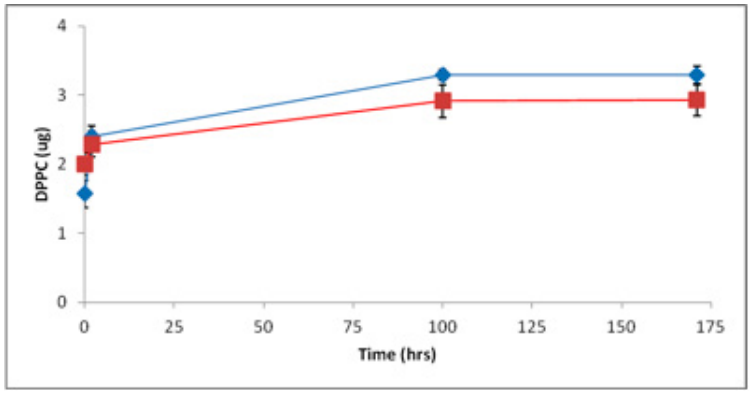

Figure 1. Direct counts of sorbed DPPC on AO (red squares) and PV (blue diamonds) lenses as a function of time following sorption. Mean and 95% CI, n>8.

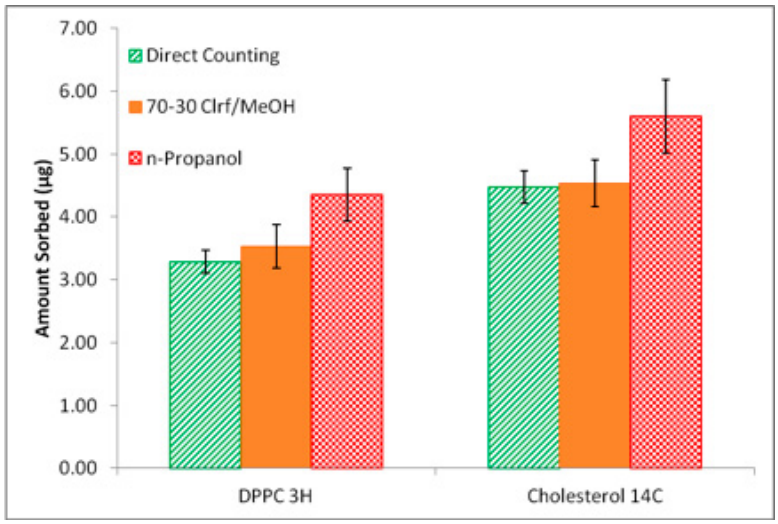

Figure 2. Comparison of various extraction methods on Acuvue Oasys lenses. Mean and 95% CI, n>12.

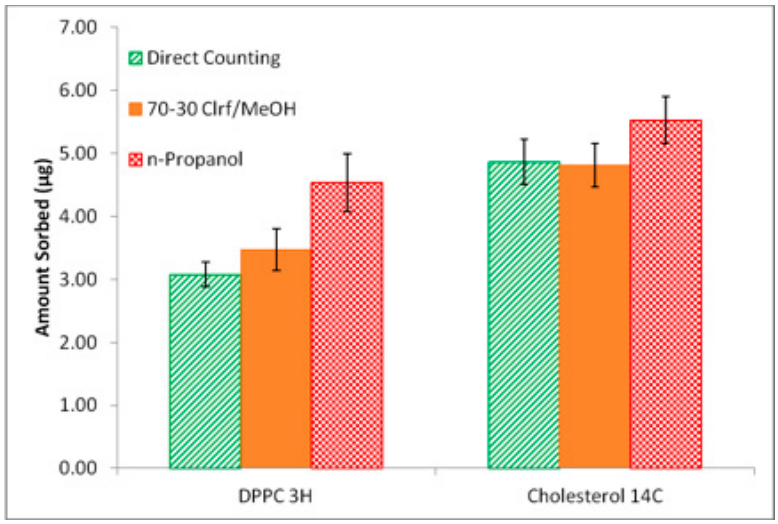

Figure 3. Comparison of various extraction methods on Pure Vision lenses. Mean and 95% CI, n>12.

Lipid Sorption of Contact Lenses Continued on Page 11

Lipid Sorption of Contact Lenses continued from Page 10

Because the total amount of 3H and 14C in a vial containing a lens cannot increase with time, these data indicate that the labeled lipids are becoming more accessible to the scintillation fluid over time. Our observation is that the SiHy lenses swell in the SF, which is known to contain hydrophobic components[6]. Thus at longer times, some emissions into the lens interior might be captured by SF.

We did experiments in which the vial of SF with a lens was counted, and then the lens was removed and the vial counted again. The cpm values were slightly lower, indicating that radiolabeled lipid had desorbed from the lens into the SF. This probably occurs over hours (the swelling in SF occurs over minutes) and may contribute to the increase in cpm over hours.

After lenses were soaked in SF for 7 days, they were removed from the scintillation fluid and cleaned as carefully as possible, and then placed in a new vial of SF. There were counts significantly above background from these vials, indicating that not all of the radiolabeled lipids had desorbed from the lenses in 7 days, even though a plateau in cpm counts was attained. Thus there was no way of knowing what counts were from lipids retained within the lens and inefficiently counted by the SF, and the lipids that desorbed from the lens.

All these factors lead us to reject direct counting as an accurate measurement of lipid sorption to contact lenses, at least when the lipids are labeled with beta emitters. Direct counting could possibly be used if enough preliminary experiments were done to establish the counting efficiency (compared to some exact method), but this efficiency would have to be determined for every new lens chemistry and lens thickness. Alternatively, lipid sorption might be determined using gamma emitters such as 125I and 131I provided that lipid species can be iodine-labeled without materially affecting their physical properties.

Chlf/MeOH Extraction and Counting

The extraction in 70% Chlf/30% MeOH was done to simulate extraction of lipids from SiHy lenses in chromatographic and other non-radioactive methods[1-3,5,7]. Specifically we wanted to know if the Chlf/MeOH extraction was really removing all the lipid from the lens. In our technique, the labeled lens was placed in70% Chlf/30% MeOH for 2 hours and the solution was sampled. We found that Chlf interfered with the scintillation of the SF, so we dried the sample in a vial, and then solvated the sample with 3 mL of n-propanol. The lens that was extracted for 2 hours was then placed in a fresh vial of SF and directly counted. There was significant residual radioactivity, indicating that the extraction procedure did not adequately extract the radiolabeled lipid. Because direct counting is not quantitative, we could not calculate the extraction efficiency from this experiment alone. However, comparison with the n-propanol extraction procedure suggests that Chlf/MeOH extraction is about 80% efficient in a single stage using this technique (see Figs. 2 and 3). As with direct counting, one could perhaps use this technique quantitatively, if enough preliminary experiments were done to establish the extraction efficiency for each lens chemistry. It is unknown if different surface treatments on the same underlying lens would affect the extraction efficiency. Another disadvantage to the technique is that the Chlf interferes with the SF and must be removed before scintillation counting, introducing another step in the procedure.

It is noteworthy that Chlf/MeOH did not extract all the radiolabeled lipid. The SiHy lenses swelled significantly in this solvent, supposedly allowing access of absorbed lipid to the solvent. However, this slightly polar solvent may not have been sufficient to elute these hydrophobic lipids from highly hydrophobic segments of the SiHy polymer. It might be possible that during lens swelling by the solvent, lipids were carried even deeper into the interior of the lens. Whatever the cause, our observation that Chlf/MeOH extraction is not complete casts some concern on data in the literature obtained from extractions of this type. One publication indicates that a 50/50 Chlf/MeOH extraction for 3 hours has an extraction efficiency of about 90% for CH from PV lenses[3], while another 70/30 Chlf/MeOH extraction showed an efficiency of about 81% for CH[7]. Thus our results are consistent with previous results. If extraction efficiencies are unknown or not employed, the published data may have underestimated what was actually sorbed by a SiHy lens.

n-Propanol Extraction and Counting

Because the Chlf/MeOH extraction was not quantitative, we also

Lipid Sorption of Contact Lenses Continued on Page 12

examined n-propanol extraction which has been shown to quantitatively extract dimyristoylphosphatidylcholine (DMPC) from SiHy contact lenses in 3 sequential extraction stages[8,9]. The first two stages of n-propanol extraction removed measureable amounts of DPPC and CH from the lenses, while the amount removed in the 3rd stage was barely above background. After 3 extractions, the lenses were placed in SF for direct counting, and only background readings were obtained, suggesting that the lenses had been thoroughly extracted. This is consistent with extraction procedures on other types of SiHy lenses[8]. The amounts extracted in each stage were summed and are presented in Figures 2 and 3. These amounts are the highest of the three quantitation techniques, further validating this technique.

We calculated the efficiency of the extractions in the first 2 stages, the first stage being about 95% efficient and the second stage being about 90% efficient in removing residual DPPC. If 90% efficiency is also applied to the 3rd stage, then the 3-stage method results in an overall extraction of 99.95% of the sorbed lipids, which is well within the error attributed to the scintillation counting itself (1%) and the variability between lenses. The main disadvantage of the triple n-propanol extraction is that it is laborious and tedious; however it yielded the most reliable results. Another advantage over ChIf/MeOH extraction is that the n-propanol does not interfere with the scintillation counting, so it does not need to be removed; a sample of lipid extracted in n-propanol can be added directly to a vial of SF.

Sorption on Four Commercial Lenses

Having established that triple n-propanol extraction was most reliable to quantitate sorption, we proceeded to measure the sorption of DPPC and CH on Acuvue Oasys, Pure Vision, Air Optix and SofLens. The first three of these are SiHy lenses, and the last is a conventional hydrogel based on poly(HEMA). The SofLens behaved differently during the n-propanol extraction in that these lenses did not swell in n-propanol and had a tendency to stick to themselves and to the sides of the glass container in n-propanol. While all SiHy lenses swelled in n-propanol, the edges of Air Optix lenses did not flare, as did Acuvue Oasys and Pure Vision lenses. Additionally, the Air Optix lenses were more robust (did not break in pieces) than the other SiHy lenses.

Figure 4 shows the sorption on these lenses. The first thing to note is that there is no statistically significant difference between Acuvue Oasys (senofilcon) and Pure Vision (balafilcon) lenses for either DPPC or CH sorption. This is consistent with other publications using other analytical methods[7,10]. However, these lenses sorb statistically greater amounts than Air Optix ($p < 0.05$) and SofLens ($p < 0.01$). The conventional hydrogel SofLens sorbs very little DPPC and CH, which is consistent with previous reports of lipid sorption on conventional hydrogels[11]. Although the Air Optix is a SiHy material (lotrafilcon), it sorbs much less DPPC and of CH (only about 1 µg/lens) than the other SiHy lenses. This also is consistent with prior studies in which lotrafilcon materials sorbed less than balafilcon materials in worn lenses[2].

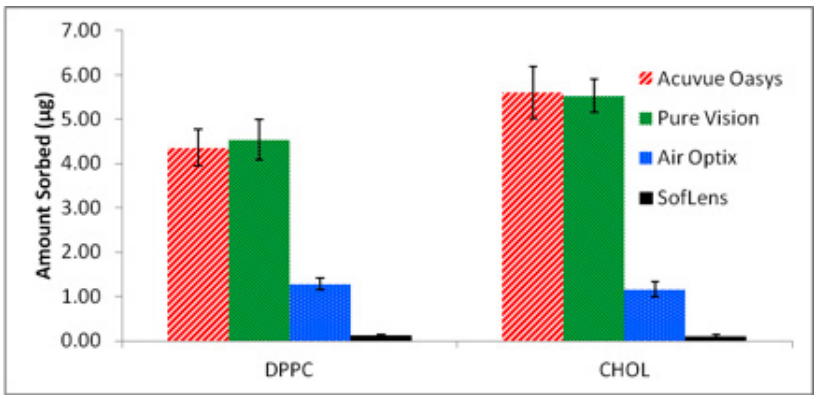

Figure 4. Amount of DPPC and Cholesterol sorbed on four lens types. Mean and 95% CI, n>12.

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Giving Hydrogels Backbone: Incorporating Physical Architecture into Soft Biomaterials

by Scott Zawko and Christine E. Schmidt, Department of Biomedical Engineering, The University of Texas at Austin

Each year the waiting list for donor organs grows longer. To meet the demand for donor organs, researchers in the field of regenerative medicine are developing man-made constructs that combine polymer scaffolds with living cells that can replace the function of damaged organs. An ideal tissue-engineered construct should possess chemical, biological, and structural features that match the tissue to be replaced. In the most general sense, tissues are composed of water trapped within a matrix of proteins and polysaccharides. To create similar materials engineers have focused on hydrogels, which, like natural tissues, are composed of water and macro-molecular components, exhibit soft mechanical properties, possess open pores for protein diffusion and cellular infiltration, and are permeable to oxygen. What hydrogels lack, however, is a three-dimensional architecture that mimics the physical structure and complexity of native tissues. Our laboratory has explored several methods to create architecturally biomimetic hydrogels.

Freeform fabrication offers the ability to create patient-specific 3D scaffolds that match the shape of a particular defect site. Our lab, in collaboration with Dr. Shaochen Chen's group, has used this technique to fabricate tubular hydrogels for nerve regeneration[1]. Each hydrogel was patterned with axial pores analogous to the basal lamina tubes found in native nerve tissue (Figure 1). The purpose of these hydrogels is to fill gaps (5-30 mm) between the ends of severed nerves and thereby guide regenerating axons from the proximal nerve end, across the gap, and to the distal nerve end. Nerve grafts with this type of physical guidance have a significant advantage over grafts without biomimetic architecture[2]. To construct these scaffolds the desired 3D shape was rendered in modeling software and then subdivided into a series of 2D cross-sectional images. Next, an aqueous precursor solution of a photoactive derivative of hyaluronic acid (HA), a primary structural component of human nerve tissue, was prepared. The series of 2D cross-sectional images were then projected, one at a time, via ultra-violet light, onto thin layers of the HA precursor solution. Exposure to UV light transformed the precursor solution from a flowing liquid to a hydrogel. By this method constructs several millimeters long can be fabricated layer by layer to any arbitrary structure incorporating any number of pores in any desired geometry.

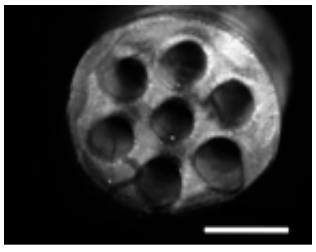

Figure 1. Cross-section of a multi-lumen hydrogel of photocrosslinked hyaluronic acid prepared by freeform fabrication. This hydrogel was embedded with fluorescent microparticles to permit epifluorescence imaging. Scale bar is 1 mm. From Suri et al., 2011[1]

The structural components of native tissues are primarily polysaccharides and proteins. Polysaccharides, such as HA, are high molecular weight polymers that bind water, thus creating water swollen matrices with compressive strength. A meshwork of proteins suspended within this watery matrix provides sites for cell anchorage and guidance. To create analogous materials our lab has partnered with Dr. Jason Shear to apply a technique, direct-write photofabrication, to embed 3D protein microstructures within HA hydrogels[3]. The microstructures can be fabricated in any arbitrary shape, like a spaghetti strand in Jell-O, or zig-zags, spirals, and corkscrews (Figure 2). These structures present both chemical and topographical cues at an impressive 0.5 µm resolution which is comparable to the diameter of a single neuronal axon or dendrite. By this method we demonstrated, for the first time, the ability to guide hippocampal neurite growth along arbitrary paths in three dimensions.

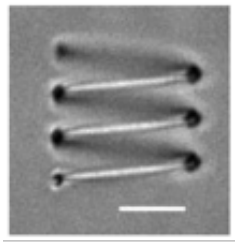

Figure 2. Direct-write photofabrication can create structures on three independent axes in tandem. This protein helix is embedded in a hyaluronic acid (HA) hydrogel and has three revolutions with a 10 µm periodicity. Scale bar is 15 µm. From Seidlits et al., 2009[3].

The complex branching patterns exhibited by native tissues, such as the microvasculature, are particularly challenging to replicate in hydrogels. To address this issue we developed an innovative crystal-templating technique using urea as an in situ crystallizing porogen[4]. Under the right conditions urea can yield highly dendritic crystals within HA precursor solutions. The

Giving Hydrogels Backbone Continued on Page 14

crystal growth compresses the polymer within narrow interstices among the crystals thus shaping the polymer into fibers (Figure 3). The photoactive HA polymer is then crosslinked around the crystals using a rapid and non-invasive photocrosslinking method. The urea is easily washed away with water leaving behind a hydrogel with a unique dendritic porous architecture.

Despite recent advances, most tissue engineered constructs are not yet ready for application to human patients. While the technology continues to progress toward human trials it is currently useful for creating model systems for in vitro testing of cellular responses to topographical cues. These experimental models are an opportunity to test and refine the fabrication methods described here and to discover the architectures to which cells best respond.

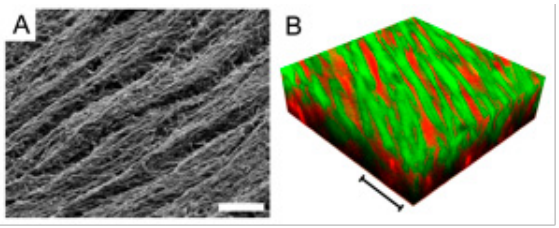

Figure 3. HA hydrogels with microarchitecture created by urea crystal-templating. (A) Scanning electron microscopy depicting the surface of a crystal-templated hydrogel. (B) Confocal microscopy of a crystal-templated hydrogel (red) perfused with protein (green) demonstrating that protein diffusion is restricted to the pore network. Scale bars are 20 µm. From Zawko and Schmidt, 2010[4].

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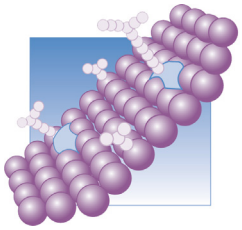

Surfaces in ***Biomaterials*** Foundation

European Regulation Update

By Phil Triolo, SurFACTS *Regulatory Editor*

For those of you procrastinators who have CE-Marked devices that have been found to meet the MDD requirements by demonstrating conformance with IEC 60601-1, 2nd edition, and have not yet retested your devices to meet the requirements of the 3rd edition which came into force on June 1, 2012: the Notified Bodies have issued a document that provides answers to questions frequently asked about the need to comply with the requirements of the 3rd edition. It can be found by clicking here.

As currently CE Marked devices do not suddenly become unsafe when a new standard comes into force, the most frequently asked question (and perhaps the most reasonably asked) is Question 3.1.2 in the above-referenced "Working Document": "When the medical device is not modified, and the regulation does not change, why do I have to provide different evidence with a new harmonized standard?"

Answer 3.1.2 invokes the state-of-the-art clause in Essential Requirement 2 of the Medical Devices Directive (MDD 93/42/EC) to conclude that conformance with the new requirements of EN 60601-1 3rd edition need to be met:

> The MDD 93/42/EC and AIMDD 90/385/EC require Medical Devices to conform with the essential requirements as given in the Annexes I. Compliance with harmonized standards evokes the presumption of conformity with the essential requirements covered. If such a standard is now no longer harmonized, because it was superseded by a revised version, the manufacturer can no longer rely on the presumption of conformity by using the superseded standard.
>
> Even if the manufacturer does not use harmonized standards for demonstrating that the medical device meets the legal requirements, *the mere fact that a harmonized standard is replaced by another harmonized standard, indicates that the state of the art has changed and that additional effort and evidence will be needed.* [Italics added]

This state-of-the-art requirement is mentioned in ER 2 of the MDD.

> Note that this does not constitute an obligation to use the latest edition of a standard, because the application of harmonized standards is voluntary but the Technical Documentation shall contain the justification for not using the standard and how the solution used by the manufacturer provide[s] at least the same level of safety and performance as if the harmonized standard would have been used.

Justification for not meeting the revised standard requirements yet still providing the same level of safety and performance for an existing IEC 60601-1 revision 2-compliant device may not be easy, and acceptance of the justification is at the discretion of the Notified Body. However it is suggested that justification would include identification of the differences between the old and new versions of the standard and an analysis of the risks posed to patients, end users, and the environment by not meeting the new requirements. Further, documentation of a review of complaints for your device and equivalent devices on the market (clinical experience) and an updated review of the current, relevant literature to validate whether or not clinical safety and performance requirements for the device are being met could be included.

A lack of significant complaints and lack of any reports in the relevant literature for hazards that could result from the failure to meet the requirements of the new standard could demonstrate that the same level of safety and performance is achieved even though the new standard requirements are not being met. Note that this analysis could take the form of a fully updated Clinical Evaluation Report, and, in any event, must be included in the relevant Technical File or Design Dossier.

Several MEDDEVs (European Commission guidelines relating to medical devices directives — the MEDDEVs aim at promoting a common approach by Member States, manufacturers and Notified Bodies and are carefully drafted through a process of consultation with various interested parties. These guidelines are not legally binding. An alternative approach may be possible for meeting directives requirements...) have also been recently published or updated. A listing of these and a brief summary of their contents or changed contents follow:

MEDDEV 2.12/1 Medical devices vigilance system (revised March 2012, came into force 15 June 2012), available here. See also the Manufacturer Incident Report (MIR) form; How to Use the MIR form; and Field Safety Corrective Action (FSCA) report form; Periodic Summary Report form (Periodic summary reporting is an alternative reporting regime that is agreed between the Manufacturer

European Regulation Update Continued on Page 17

and the National Competent Authority for reporting similar incidents with the same device or device type in a consolidated way where the root cause is known or an FSCA has been implemented) available here; and a list of current contact points for reports. The MEDDEV and forms provide a means of providing electronic input into a European database (EUDAMED). There are some reporting changes that need to be reviewed and implemented into your quality system to assure compliance.

MEDDEV 2.12/2 Post Market Clinical Follow-up (PMCF) studies (new MEDDEV), available here is not applicable to IVDs (In-Vitro Diagnostic Devices). Significantly, each Notified Body is now required to "verify that PMCF is conducted when clinical evaluation was based exclusively on clinical data from equivalent devices". So, if the "literature route" was used to demonstrate the clinical safety of a device in a Clinical Evaluation Report, PMCF studies must be planned and carried out. If no PMCF studies are planned, justification for not conducting the studies must be provided to your NB who must agree with your justification.

MEDDEV 2.5/10 Guideline for Authorized Representatives (NEW – January 2012), available here [only applies when the manufacturer is not located in an EU country] Most importantly, this MEDDEV provides guidance on what contents to include in signed agreements between Authorized Representatives (ARs) and manufacturers, documents that an AR is required for devices subject to clinical investigations conducted in the EU, and provides additional information on the role ARs play in pre-and post-market activities and the relationship between ARs and manufacturers in these activities.

MEDDEV 2.1/6 Qualification and Classification of standalone software (NEW – January 2012), available here. This MEDDEV provides guidance on how to determine if software is a standalone device, amongst other suggestions, clarifications, and interpretations.

MEDDEV 2.14/1 IVD Medical Device Borderline and Classification issues. A guide for manufacturers and notified bodies (UPDATED – January 2012), available here. Most importantly, provides guidance on how to determine whether a device is an in-vitro diagnostic or general laboratory device, and whether or not the device is governed by the IVDD or MDD.

MEDDEV 1.14/4 CE marking of blood based in vitro diagnostic medical devices for vCJD (Variant Creutzfeldt-Jakob disease) based on detection of abnormal PrP (Platelet-Rich Plasma) (NEW – January 2012), available here. This MEDDEV identifies basic quality requirements for assays for detection of vCJD (Mad Cow Disease).

A more thorough discussion of some of these MEDDEVS may be found here. A complete list of all MEDDEVs is available here.

Happy reading!

Innovations in Biotechnology Across Global Markets

By Hans Ole Klingenberg, Product Director at Novozymes

Introduction

Biotechnology-based innovation has increased rapidly in recent years. Amongst the countries striving in this field, China is one of the most prominent, having seen double-digit growth over the past few years. The country now serves as a promising location for biotech companies looking to expand their business. With a long running business history and over 20 years experience in running and operating facilities in China, Novozymes has achieved a position in which it can reap the full advantages of China's expanding biotechnology market. In line with this, Novozymes has recently become the first western biotech company to open a wholly owned GMP facility for manufacturing a biological API in China. The new site is a dedicated facility for the production of Novozymes Hyasis®, hyaluronic acid (HA), in compliance with Q7 cGMP (current good manufacturing practices) for biomedical and pharmaceutical applications. Novozymes' global biopharma operations group now spans facilities in China, Denmark, and the UK.

HA, also known as hyaluronic acid, is a naturally occurring polysaccharide which is distributed widely throughout connective, epithelial and neural tissues in the human body. As a component of the extracellular matrix, HA provides structure to tissues including skin and cartilage, and is therefore well suited to a wide range of pharmaceutical and medical device applications. Novozymes Hyasis® is produced using an innovative manufacturing process that raises the current standards for safety, consistency and performance. It is the world's first and only recombinant source of hyaluronic acid based on fermentation of the safe microorganism *Bacillus subtilis*, a non-pathogenic host used by Novozymes in the manufacture of several products with Generally Recognized as Safe (GRAS) status by the US Food and Drug Administration. The process uses minimal media, no animal-derived raw materials and a proprietary water-based technology that eliminates the use of organic solvents, offering a high degree of purity, increased safety, batch-to-batch consistency and stability in large scale.

Operating on Novozymes' key strategic site for production in China, the new HA facility shares its location with Novozymes China's current operations. With over 1,000 employees, Novozymes China now represents approximately 20% of Novozymes' global staff, making China Novozymes' second largest regional market. The new HA facility fully conforms with the ICH Q7 Guidelines for API manufacturing and enables all regulatory requirements for using HA in drug products to be met.

Deciding Factors

An extensive selection process was conducted by Novozymes when choosing the site's location. Considerations regarding current presence, resourcing, and future growth were instrumental in the final location decision.

An already established presence, teamed with Novozymes' existing operations in China, were key factors in the selection of location. The company wanted to take advantage of the team and processes already established by its existing operations in China and leverage this activity to function on a larger scale. By taking the logical next step of building on already existing operations, potential complications associated with developing a completely new site, such as reduced ownership and quality control, were avoided.

In addition, a further consideration was access to resources. The number of trained scientists living and working in China now reaches over 55 million, meaning that there is a wealth of locally sourced engineers, operators and biotech trained production individuals providing a significant resource for recruitment.

Another critical factor in selecting the location of the facility was the perceived likelihood of future growth within the Chinese domestic biotech market. Recent business intelligence reports predict that the Chinese biotechnology market is expected to grow at a CAGR of 23% from 2007-2012. To fully capitalize upon this

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expanding market, a local presence is key. The increasingly strong presence of Novozymes within the region ensures that the company is able to expand and develop in line with growth in the Chinese biotech market.

Stringent Quality Assurance Measures

Regardless of location, strict measures are carried out at all Novozymes facilities to ensure that the quality of products is upheld to the company's global quality standard. To avoid quality control issues, companies must be fully invested in every aspect of a site, with full transparency and ownership ensured. Designed and built by NNE Pharmaplan, Novozymes' facility has been designed to ensure rigorous control of all activity within the site alongside full compliance with cGMP regulations, ensuring high quality products are manufactured. For further assurance of quality control, Novozymes runs its own QC labs on site which test and monitor all incoming and outgoing materials. The site also sources compendial grade (USP grade) raw materials and selects internationally recognized vendors, using a risk-based approach. A dedicated team with extensive experience of running cGMP facilities in large international companies oversees this activity.

Manufacturing Improvements and Decreased Business Risk

The opportunity to break new ground and look at novel ways of manufacturing high quality ingredients spurred Novozymes' decision to implement cGMP operations in China. The technology utilized at the facility is a significant departure from traditional methods, which require the use of ethanol for recovery. Instead, Hyasis is based on a Bacillus-derived–HA technology, using water-based techniques in the recovery process and replacing conventional solvent-based techniques. Novozymes' unique spray-drying method produces a very fine powder composed of microparticles which delivers unique performance benefits to customers.

Characterized by a unique set of properties, Hyasis has a well-controlled and reproducible molecular weight, low polydispersity and long shelf-life. It is easy to dissolve and filter during manufacturing, and its exceptional heat stability permits autoclaving as a sterilization option. Hyasis can be used in a broad range of applications in the pharmaceutical sector for improved drug delivery, as well as in medical device application areas including: ophthalmology, dermatology, osteoarthritis, dermal fillers, adhesion prevention, coating, and wound healing.

Recent years have seen the popularity of animal-free ingredients increasing, with regulatory authorities enforcing stringent quality measures on products to improve safety, particularly with potential contamination risks from pathogens such as viruses in animal-derived ingredients. Hyasis is 100% free of animal-derived raw materials and organic solvent remnants, making it an ethical and safe choice for the production of biopharmaceutical products and devices.

Hyasis offers manufacturers of HA-based medical devices or pharmaceuticals the opportunity of cost savings in manufacture as well as a decrease in business risk due to lower risk of adverse events.

Closing Thought

The decision to locate the new Novozymes' HA facility in China was made after extensive assessment of various factors including current presence, resourcing, future growth and investment cost. Following a thorough investigation, China was deemed as an ideal location for Novozymes' overall strategic expansion. Risk of quality control in

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this location was assessed by the company and judged as low due to the company's implementation of significant control measures to ensure that standardized quality levels are upheld. Throughout this process Novozymes' primary focus has been to deliver a more consistent source of HA, offering a high degree of purity whilst decreasing regulatory burden. This will assist Novozymes' customers to develop improved medical devices and drugs to provide increased patient benefits.

For further information on Novozymes' Hyasis, please visit www.hyasis.com.

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About Novozymes

Novozymes is the world leader in bioinnovation. Together with customers across a broad array of industries we create tomorrow's industrial biosolutions, improving our customers' business and the use of our planet's resources.

With over 700 products used in 130 countries, Novozymes' bioinnovations improve industrial performance and safeguard the world's resources by offering superior and sustainable solutions for tomorrow's ever-changing marketplace.

Novozymes' natural solutions enhance and promote everything from removing trans fats in cooking, to advancing biofuels to power the world tomorrow. Our never-ending exploration of nature's potential is evidenced by over 6,000 patents, showing what is possible when nature and technology join forces.

Our 5,000+ employees working in research, production and sales around the world are committed to shaping business today and our world tomorrow.

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