When the Münchenstein railway bridge – a bridge built by Gustave Eiffel (who later built the Eiffel Tower in Paris) – came crashing down in Switzerland’s worst railway accident in 1891, EMPA famously investigated the cause of the collapse.

Now, the institute has switched gears toward research on new materials, especially in the areas of energy, building, the environment, pollutants, and medical safety applications — including in the field of nanomaterials or nanostructured materials. While the field of nanomaterials may offer many promising solutions, some challenges must be tackled first.

EMPA collaborates with over 500 industry partners, such as Johnson & Johnson, to develop medical devices ranging from artificial tendons, carbon-coated hip joints, to a cell-seeded polymer graft to regenerate the myocardium after an infarction. EMPA’s main area of focus is in biology, especially tissue regeneration, and by developing wound healing and bone substitute materials. EMPA also works with hard materials, such as implant surfaces, and human stem cells — isolating bone marrow stem cells and testing these surfaces with human stem cells. EMPA is particularly active in the field of medical textiles. Last but not least, EMPA is involved in the evaluation of these biological tools and the toxicity and safety aspects of these materials.

In the healthcare world, nanomaterials may play a role now or in the future in the realms of textiles, biomedical and health care materials … ranging from sunscreen, to drug delivery, to dental ceramics, to cancer treatments.

A simple and obvious example is the use of nano titanium dioxide and nano zinc oxide in sunscreens. These are very efficient absorbers/reflectors for UV-light and thus, protect the skin from DNA damage and the induction of skin cancer by UV radiation. This is not really a medical application but a precautionary measure for human health, which additionally saves the environment from the burden of a multitude of chemicals that are normally used to absorb UV-light, exposing aquatic organisms in the process.

In an example of how nanoparticles may be used in cancer treatment, MagForce in Berlin isolated iron oxide nanoparticles in an aqueous solution, and developed a method to inject them into inoperable tumors in the brain. The particles heat up through an alternating magnetic field, and heat the tumor to hopefully kill the tumor. This is a direct treatment with nanoparticles other than a pharmaceutical. It is considered a medical device, because it results in a physical rather than a biological effect.

**Schematic Overview of Implant surface functionalization and the interaction with cellular systems**

EMPA tends to concentrate on nano-structured materials, such as surfaces for implants, taking simple methods already developed and creating more complex, advanced biological methods that simulate in vivo situations to evolve toward better conclusions for implant surfaces. By altering the structures and manipulating the roughness of the surfaces, researchers try to find a topography that can influence the behavior of cells; for example, the differentiation of bone marrow cells.

**Revcel**

Another example is Revcel, a wound-healing, polyurethane-based surface treated foam. Introduced into a chronic wound, it is biodegradable. Neighborhood cells move in to the scaffolding and close the wound. The foam degrades and goes away … but you have to consider the safety features of the material that degrades.

**Advanced plasma polymer coatings for biomedical applications**

Dirk Hegemann and colleagues at EMPA created an advanced plasma polymer coating used for heart muscle regeneration. Isolated cells are integrated on a polymer matrix, and then the engineered tissue is implanted back into the heart, in the hope that the muscle will regenerate. First, an electrospinning procedure produces biodegradable nanofibers. Then, on top of the surfaces of the nanofibers, to attract the surrounding cells and
support their growth, plasma polymers containing nitrogen are applied to attract cells. However, if you increase the nitrogen concentration, it could adversely affect the surfaces.

**Stability of plasma polymers**

To demonstrate this effect, Hegemann delivered four different ratios of the nitrogen source to the carbon source. If these surface-coated materials (and keep in mind, the coating is very thin: less than 10 nanometers thick on the surface) are put in water or left exposed to the air, some ammonia that is not bound in the plasma to the surface material and not integrated very well into the material is released from the surface. It always comes down to the same concentration; you cannot increase the concentration of the nitrogen source above this 2% volume. This release of nitrogen will kill the cells in the MTT assay, and after storage in air the cell viability comes back. The degradation of the plasma polymer product, especially the release of the nitrogen, is toxic in this case.

Some of the earlier presenters set the scene for unique challenges in working with nanomaterials for in vitro tests, but this takes us to another, serious problem that we have with nanomaterials: They have different chemical, physical effects because of their size, and we have to realize that probably this is true in biological systems as well.

**Side effects of nanomaterials**

Although human skin is a very effective barrier for nanomaterials, they can be taken up through inhalation, ingestion and medical applications such as intravenous or intraperitoneal injection, and they may penetrate into the bloodstream and may be distributed all over the body, finding target organs where they possibly may trigger side effects — including the induction of oxidative stress and inflammatory responses.

**Interferences often neglected**

Krug and his colleagues, in a paper published in *Nano Letters*,¹ showed that scientists drew false positive results working with carbon nanotubes. The carbon nanotubes bind the dye of the MTT assay, and during the extraction and measurement procedure some dye is extracted as well — and the nanotubes are lost, leaving too small a number for the cells to survive.

Their findings strongly suggest the need to verify cytotoxicity data with at minimum a second method, as suggested by the ISO standards as well. At a minimum, at least a second independent test system is needed for this new class of materials (nanomaterials). Furthermore, reference materials should be used, as well as harmonized or standardized protocols should be used. If you have an idea about the size of the effect that you measure, you’ll find a huge amount of papers without any controls, and without any positive controls.

For example, oxidated stress measurement regarding nanomaterials often uses the fluorescence TCF assay that results in a one-fold, two-fold, or three-fold increase. But what does it mean if you have no control, and no sense of the cells can do if they are induced to produce such oxidative stress related effects. This is just an idea of the difficulties faced in the field.

In recent years, there has been a dramatic increase in papers related to nanotoxicology, with more than 2000 papers in 2012. How relevant are these papers? Hristozov et al, in *Nanotoxicology* 2012, published an insightful paper that demonstrates the problems. For titanium dioxide and zinc oxide – the two most prominent nano-sized materials for use in cosmetics and other applications – there were nearly 300 papers that delivered toxicological information for these two materials, and around 40 papers with valid data on the physical and chemical properties of these materials. Next, Hristozov’s team looked at how useful these papers are for toxicological judgment, evaluation. For physical and chemical properties, nearly no papers remained. In the case of

toxicological information the number of papers that offered toxicological information with usable data that can help evaluate toxic effects of these materials dwindled to about 30 percent. Therefore, who can judge which papers fulfill the criteria, if 90% will not fulfill the criteria? And, who will come to a conclusion on this level of data sets? Therefore, four years ago we set out to establish a platform to assess the nanoparticle toxicity in vitro, especially for four different endpoints (viability, inflammation, genotoxicity, and oxidative stress); to integrate the pathways of toxicology. Which activities related to nanoparticles should receive a closer look? This project, funded by industry and the Swiss government, is now in the phase of writing up SOPs to harmonize the protocols.

Krug and his colleagues also wanted to deliver more guidance on how regulators may judge nanomaterials; considering, for example, whether there might be a tool that can be used (like TCCs) to help judge, in the absence of enough data, the use of nanomaterials in consumer products.

To convince the regulators, EMPA and other groups launched a voluntary alliance, the International Alliance for NanoEHS (IANH) for the harmonization of protocols, including 11 labs in the US, Europe, and Asia generating data. The first data published focuses on the characterization methods of nanoparticles. Most of the papers do not even deliver the size of the particles they used for the studies; that is a huge gap in the characterization data. To address that shortcoming, IANH offered suggestions for common, harmonized protocols to measure the nanoparticles. The first effort to develop a protocol for a toxic effect, however, encountered problems. Starting with the very simple, five-step MTS assay, IANH delivered materials, and participating labs would then deliver the results. Seven labs in a round robin, using the same protocols, did not come to the same results. Far from achieving harmonization, there were severe discrepancies between the curves. (Need to include raw data slide, #21)

What happened? In 2011 L. Locascio, in “Variables Associated with in vitro Toxicity Testing,” highlighted variables to consider if you investigate nanoparticles. The first set of variables regards the nanomaterial samples themselves … the composition, the sample purification. Most nanomaterials are not sterile; they are not packed sterile, and they all are contaminated with endotoxins. Nearly 90% of the researchers deliver data that are not free of endotoxins … and they, of course, publish on inflammatory effects. The next set of variables is associated with the toxicity test assays. Most people disregard interferences. Nanoparticles usually have different absorbance, color, etc.; these must be determined upfront with appropriate positive and negative controls. Depending on the desired endpoint, there are a wide variety of toxicology tests each with their own set of endpoints.

Despite characterization SOPS, harmonized protocols, and biological models, for IANH’s own voluntary round robin the results were not valid, even though all the labs used all the same materials, protocols, and cell lines. In the end, it was discovered that masters students and inexperienced PhDs did the experiments, which would have been performed by the best technicians in a round robin in the medical area. Therefore, IANH decided to create another round, this time selecting five institutes (NIST, KRISS, JRC, EMPA, NANOTEC). The results turned out great; the EU institutes were very close. The Asian and US numbers were a little different because they were not allowed to import the same serum that the EU institutes used. The serum is the only thing that was different in these tests, and you see that the serum does have an influence here.

New models for nanoparticles
A solid understanding of barrier systems, such as the blood-air and blood-brain barrier, is very important to judging the effects of nanomaterials. To determine if the placenta is an efficient barrier for nanoparticles, EMPA studied real human placentas obtained from clinics. They showed good difference between smaller particles, which can appear on the inside of the fetal surface, while larger do not. This was the first evidence that nanoparticles can go through such human barriers.

These studies reveal a number of challenges inherent in nanotoxicology:
We have to correct the methodologies, and the study designs
- Use of SOPs or adapted guidelines; harmonized protocols
- Dose or concentrations are an issue
- Measurement of nanoparticles *in situ*
- Selection of the biological models
- Comparison to the correct controls; this is a missing link
- Conclusions and stories

**Dose and Concentrations: Dosimetry puzzle for risk assessors**

- Dose metrics are very important, especially for nanoparticles, because you can use different dose metrics. You can use the number of particles, the specific surface area, and so on.
- Normally chemicals are very well-distributed if you deliver this to cell cultures, but nanoparticles behave differently. The concentration of the amount of particles that cells see in a certain system is not dependent on concentration of the suspension – but it is dependent on amount of the suspension as well.
- So the volume you add to the cells is another very important point to judge the concentration that the cells see, because in a medium containing serum the nanoparticles start to agglomerate, they become larger and larger, they form sediment on top of cells, and nearly 100% of the material after several hours is on top of the cells. In an example shown, the layer is 65 nanometers thick, and all nutrients and oxygen have to go through this layer. The surface of nanoparticles is very active, and binds nutrients, of course these cells lack these nutrients and will eventually die. Thus, studies highlighting cell deaths that have been published may be a result of the covering of cells with the material, and not a specific effect of a given material.

- Example: 2010, Swiss scandal that newspapers picked up; a well known researcher on inflammasome, started to investigate nanoparticles and concluded that in all consumer products that contain these nanoparticles (toothpaste, cosmetics, sunscreen — the list is long), they have the same toxicity as asbestos inhalation. The concentration of the nanomaterials he used for his THP1 cells, 200 µg/ml – leading to up to 500 mL thick layer of nanoparticles on top of the cells. Therefore, of course the cells were affected; and, because they were THP1 cells (a human monocytic cell line derived from an acute monocytic leukemia patient), the first thing they do before they die is send out inflammatory mediators.

- This can all be summed up as follows: No controls, no dose-response relationship, no characterization = no meaning.

**Toxicology in the 21st Century for Nanomaterials**

A number of investigators are publishing work on common pitfalls in nanotechnology:

- Scott McNeil and team; focuses on specific things like endotoxin, characterization residual manufacturing, sterility, batch-to-batch consistency, etc.
- Peter Hoet – Belgian who concentrated on the biological systems or cell density, assay methods, serum and solvents that have been use.
- EMPA – has published ideas on how to proceed to get some reliable test systems, calibrating reference materials; comparison to results achieved by other methods, inter-laboratory comparisons are needed to rule out errors appearing in literature.

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These efforts are all contributing to the need to build a stable body of reliable and comparable results, built on quality-management systems, validation, measurement uncertainty, and traceability.

The website www.nanoobjects.info includes information about running projects in Europe and the knowledge base, including ideas about 25 materials to date, applications, methodology, and literature criteria for inclusion on this website. The website also includes SOPs for harmonized protocols.

This demonstrates efforts to implement some ideas about the development of nanoparticles develop in our technical environment, especially regarding health, to nanodevices, the use on surfaces, surface coating, textile coating and sensors so on. EMPA/IANH protocols will be delivered to the OECD and ISO system, and hopefully our protocols can find a way there, and eventually develop harmonized international protocols in collaboration with US and Asian institutes.

Discussion
How nanoparticles are changing the field
When nanoparticles first came out and we began to see them being used, we understood that it would be a disruptive device, in the same way that iPhones were: they changed everything. In the past decade, there has been an explosion of products containing nanoparticles. However, most of these nanoparticles, like in iPhones, are bound into a fixed composite material matrix, and will not be released until the end of life. EMPA has a life science lab that is looking at what is happens at this end phase. For example, if these products are burned in an incinerator, are the particles released again? You have composite materials, surface coatings with nanoparticles, glasses coated with nanoparticles (to repel water and dust particles, making them easier to clean), cosmetics, etc. Is there a possibility to ingest or inhale these particles?

On the other hand, we have to realize that nanoparticles are not new; they were around before the atomic force microscope delivered the source for the technical term nanotechnology and the manipulation of single atoms and molecules. We’ve used nanoparticles for over 100 years, intentionally, for example as pesticides and fungicides. Titanium dioxide has been used for over 50 years in products, and we’ve experienced what nanoparticles do in terms of exposure to humans, animals, the environment, with awareness that they may deliver adverse effects. Perhaps models similar to TCC can be used to extrapolate relevant data. We have different ways of judging these particles, now we need to harmonize them internationally.

Human health endpoints and existing guidelines
As in other areas, for nanomaterials we also have to consider human health performance. Are new in vitro tests needed, or are existing OECD guidelines available that should be modified for nanoparticles? EMPA’s approach is to make use of existing protocols as closely as possible related to the guidelines, and adapt them. In Krug’s opinion, new assay systems are not needed; existing assays can be modified for nanomaterials. But we have to respect the specific properties of these materials. This integrated approach is built into the SOPs that will be available in 2014, which will hopefully encourage others who publish in this area, even outside of the IANH round robin participants, to use these ordered protocols.

Next steps for medical device working groups
A working group – ISO Working Group 17 – has been established to deal with nanomaterials in medical devices. From a safety point of view, perhaps the most important area of focus for this working group to consider is the analytics; to demonstrate that nanoparticles, if they are integrated into nano-structured surfaces, are to some extent stable in the biological system. Abrasion or other physical/mechanical treatments of these devices during their operation lifetime should not deliver more material than usual materials do. Implant surfaces, for example, experience some abrasion, and deliver particles. Even from normal implants, the particles are vulnerable in this case. The same is true for technical products; several German studies compared the abrasion process of manipulating products that contain composite materials like polymers, plastics and so on that contain nanoparticles, against those that do not contain nanoparticles. If you subject them to same manipulation (abrasion, sawing, etc.) the resulting release particles is nearly the same. You do not find the
original nanoparticles outside of the material or in the air if you analyze this very carefully; they are all bound to matrix and don’t differ from normal material.

The same may be true for medical devices, but this must be judged on a case-by-case basis, depending on what task nanoparticles fulfill in a medical device. The exposure route or the dose of exposure matter here, but the analytics and characterization are the most important points.

Tools to judge the quality of published studies
EMPÃ’s website publishes criteria used to evaluate *in vitro* studies; it is a literature criteria catalog designed to help judge the quality of a published study, not of the materials themselves … looking for characterization, biological systems, and the right controls. It can be downloaded and adapted for normal chemicals as well; but it is to judge the quality of the study.

In the context of the evidence-based toxicology work, Hartung’s team has developed a tool, the ToxRTool, which is a systematic way of assigning Klimisch scores to both *in vivo* and *in vitro* toxicology studies. It is not a perfect tool, but it is now used by the National Toxicology Program for judging existing studies (what has been published, what is the quality) as a first step toward a general tool for assessing the quality of published materials. The FDA is actually evaluating the ToxRTool, in addition to other evaluation criteria for studies published on device materials, and so far has been very impressed by the ToxRTool.