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Nanobiomaterials and Nanoanalysis: Opportunities for Improving the Science to Benefit Biomedical Technologies**

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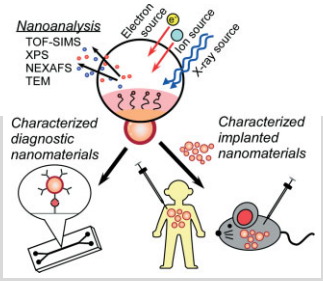

Nanomaterials advocated for biomedical applications must exhibit well-controlled surface properties to achieve optimum performance in complex biological or physiological fluids. Dispersed materials with extremely high specific surface areas require as extensive characterization as their macroscale biomaterials analogues. However, current literature is replete with many examples of nanophase materials, most notably nanoparticles, with little emphasis placed on reporting rigorous surface analysis or characterization, or in formal implementation of surface property standards needed to validate structure-property relationships for biomedical applications. Correlations of nanophase surface properties with their stability, toxicity and biodistributions are essential for in vivo applications. Surface contamination is likely, given their processing conditions and interfacial energies. Leaching adventitious adsorbates from high surface area nanomaterials is a possible toxicity mechanism. Polydimethylsiloxane (PDMS), long known as a ubiquitous contaminant in clean room conditions, chemical synthesis and microfabrication, remains a likely culprit in nanosystems fabrication, especially in synthesis, soft lithography and contact molding methods. New standards and expectations for analyzing the interfacial properties of nanoparticles and nano-fabricated technologies are required. Surface science analytical rigor similar to that applied to biomedical devices, nanophases in microelectronics and heterogeneous catalysts should serve as a model for nanomaterials characterization in biomedical technologies.

1. Introduction

Current trends to shrink the dimensionality of materials are driven by the desire to access the unique material properties and performance advantages that appear in the transition to nanometer length scales. As size diminishes to the nano-scale, certain properties of matter become scale-dependent. These include: capillary forces, optical effects/color, melting points, conductivity, ionization potential, electron affinity, magnetism, and, significantly, surface energy and reactivity.

Among unique optical, electronic, mass transfer and thermal property changes deliberately pursued in the nano-scale materials regime, the impact of increasing surface area with decreasing particle size must be considered. Sub-micrometer sized particles (e.g., nanoparticles, NPs, diameters 1–300 nm) are the most accessible and manipulated form of nanomaterials to date – over a dozen major reference texts have been published on NPs alone in the past 8 years. Consistent with this, such NPs are among the nanomaterials closest to commercial markets.[1,2] NPs are also popular in biomedical applications for in vitro and in vivo diagnostics, drug delivery systems, imaging tools, and immunolabeling.

2. Nanophase Surface Properties

Surface structure and composition, and hence reactivity, are perhaps the dominant properties in NP materials. Specific sur-

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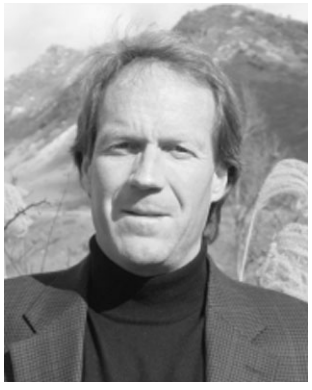
face areas for micrometer-sized particles (e.g., fumed silicas, commercial carbon blacks) are typically 60–80 $m^2 g^{-1}$, a considerable surface-to-mass ratio. Decreasing microparticle diameter to tens of nanometers increases the specific surface area up to 5 times more – an amazing amount of surface area per mass. Further, commercial CB-1 carbon black and newer single-wall carbon nanotubes – another major nanobiotechnology interest – have specific surface areas approaching 1000 $m^2 g^{-1}$. Analogous scaling effects are seen with miniaturization of surface topology, porosity, texturing, and high-density fabrication in sub-micrometer features. Hence, surface effects must also be considered a unique and very significant functional nano-property that require both control and careful characterization enroute to exploitation in specific nanotechnologies. *This is particularly true in NP applications of these materials in biomedical systems where exquisite control of the interface is required to produce specific interactions with biology.*

Such high specific surface area materials exhibit substantial challenges in controlling interfacial reactivity. Compared to bulk-phase atoms, two important, distinguishing features of surface atoms are (1) their lower coordination number and (2) their increased exposure to reactive species in the environment. These features translate to intrinsically higher surface atom reactivity than bulk atoms and manifestation of this reactivity in some usual but also other very unique ways. As particle size decreases the surface properties of the atoms dominate, leading to significant changes in particle reactivity. The relative fraction of surface atoms to bulk atoms in a structure is called *dispersion*, a quantity with a power law scaling in the nanoscale regime. While less than 1 % of a *micro*particle's atoms occupy surface positions, over a tenth of the atoms in a 10-nm diameter metal particle reside on its surface (and 60 % in a 2-nm particle!). Figure 1 shows the relationship between dispersion and particle size. In addition, depending on NP shape, even more highly reactive, lower-coordinated edge and vertex surface atoms can make up a significant fraction of the surface as particle size approaches a few nanometers. For example, metal particles in an octahedron shape have atoms with coordination numbers of 4 (vertex) and 7 (edge) that dominate their surface for diameters below 2 nm.[5] Figure 2 shows examples of octahedron and cubo-octahedron particles that contain surface atoms with coordination numbers of 9 (<111> terraces), 8 (<100> terraces), 7 (edges), and 6 (cubo-octahedron vertices) and 4 (octahedron vertices). Oxygen interactions with metallic Pt demonstrate the dramatic differences that dispersion exerts on reactivity. Low dispersion, mm-thick metallic Pt single crystals exposed to oxygen at room temperature form a layer of chemisorbed oxygen atoms on the metallic Pt crystal surface.[6] In contrast, highly dispersed, supported metallic Pt NPs used for petroleum refining are completely converted into Pt oxide.[7] This disparity



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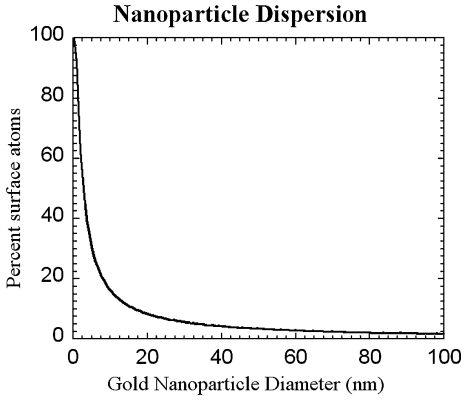

Figure 1. For gold and other metallic nanoparticles with diameters below 20 nm, the percentage of nanoparticle atoms that reside at the surface increases dramatically. This relationship assumes a face-centered cubic structure with gold atomic diameter of 0.288 nm, and that the number of atoms present in each shell is 10 $n^2 + 2$ (see Ref. [3] and [4])

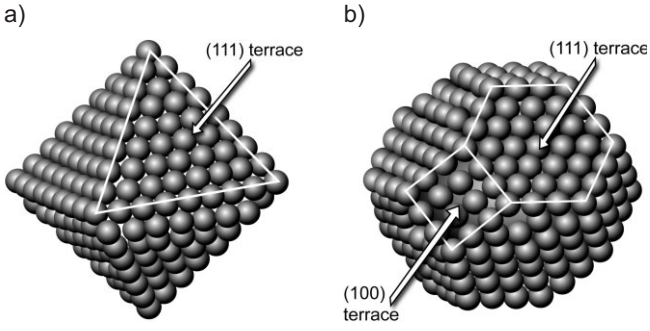

Figure 2. a) and b) Face-centered cubic octahedron (a) and cubo-octahedron (b) shapes of nanoparticles. The octahedron (a) has <111> terraces with 9-coordinate surface atoms, edges between the <111> terraces with 7-coordinate atoms, and vertices with 4-coordinate atoms. The cubo-octahedron (b) has <111> and <100> terraces with 9- and 8-coordinate surface atoms, respectively, edges between the terrace planes with 7-coordinate atoms, and vertices with 6-coordinate atoms.

distinguishes the chemical reactivity differences attributed to both nano-phase surface reactions and distinct 'bulk-phase' properties in nanomaterials.

Another testament to the unique surface reactivity of nanomaterials is their deviation from the often-applied and well-known Gibbs–Thompson relation classically describing how an atom's chemical potential (energy) in a nanophase exceeds that for the same atom in 'bulk' (i.e., material representing the extended solid phase). Recent evidence has shown that an atom's chemical potential in a NP increases much more radically with decreasing particle size than predicted from this often-used relationship.[8] This can be attributed to the increasing fractional contribution of surface energy and surface versus bulk atomic occupancy, with surface atoms less stabilized by full coordination compared to bulk, rendering them more reactive. Differences in energy between surface and bulk atoms can also lead to rearrangements of surface atoms, even for "clean" metal surfaces in ultra-high vacuum. Typically these rearrangements only involve an expansion or contraction of surface atom positions by a few percent from the nominal bulk interlayer spacing.[6,9] However, in some cases significant reconstruction of the metallic surface occurs.[9] The significance is that high specific surface area materials demonstrate enhanced, unique size-dependent surface reactivities, most of which have not been characterized or quantitated. They have proven difficult to control in high vacuum, gases, or aqueous buffers, *let alone in serum containing 70 mg mL^{-1} surfactants and surface-active proteins, or in vivo systems.* Copper particle toxicity in mice was recently shown to be size-dependent (and interestingly also gender-dependent),[10] where neither ions nor microparticles were particularly toxic, but sub-micrometer particles were acutely toxic.

The limited understanding of the differences between traditional bulk materials surface science and that for nano-phases produces daunting challenges for applications of these materials in biotechnologies. The increasingly common scenario reported in the biomedical literature involves the biotechnologist preparing and placing a very complex and poorly defined, highly reactive materials system, with its intrinsic power law-dependent interfacial properties, into an even *more complex* biological system. The result often is not definitive, elucidating, mechanistic or very good science that might inspire approaches to improving technology. *The situation begs the question about how to improve the (nano)science to best benefit such studies.* The biomedical assay approach typically employed starkly contrasts the carefully controlled ways that, for example, supported NPs are studied and handled for gas-phase catalysis experiments (i.e., for heterogeneous catalysis). As a major prime mover of surface science, the catalysis research field has a long and significant history of innovating and applying powerful surface analysis methods (e.g., X-ray photoelectron spectroscopy (XPS), low energy electron diffraction methods like LEED, Auger electron spectroscopy, mass spectrometries, etc.) to obtain detailed characterization of these NPs both prepared and analyzed under well-defined conditions. Typically this involves specialized instrumentation and standard, validated experimental protocols to prepare, store and transport samples in specific, well-defined conditions (oxidized, reduced, sulfided, etc.) for analysis (e.g., see ref. [11] and [12] and references therein). Due to the complexity of most biological environments, analogous surface science methods for NPs in complex milieu are lacking; direct translation of traditional ultrahigh vacuum (UHV) surface science methods to particles placed into aqueous, proteinaceous environments provides limited information. Reliance of the field to date on transmission electron microscopy to describe particle behavior is insufficient and misleading since it is performed ex situ after dessication, and provides little surface information. The schematic in Figure 3 shows the various stages of NP synthesis, functionalization and biological applications with characterization challenges presented at each stage.

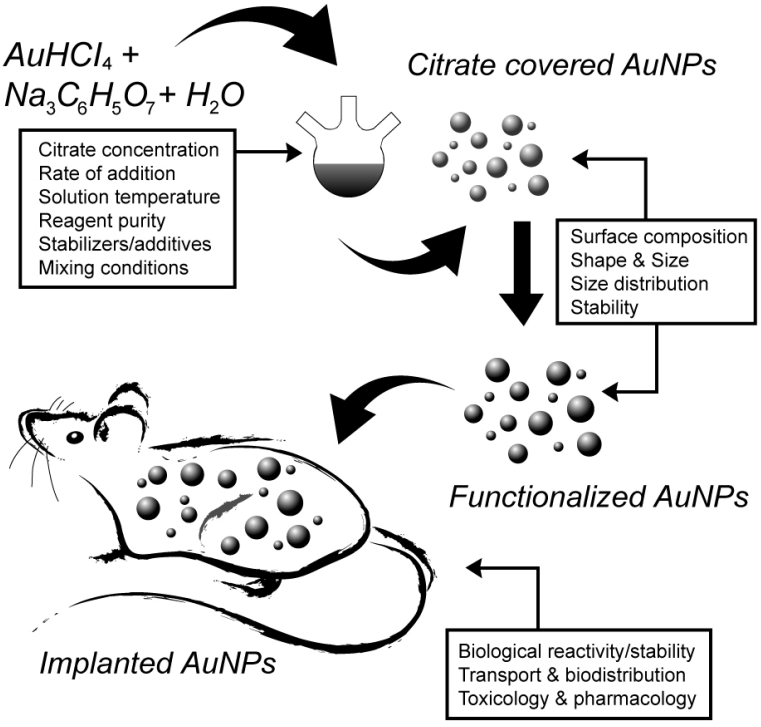

Figure 3. A schematic example of the checkpoints required in the synthesis, functionalization and biological injection or implantation of gold NPs. Aqueous citrate reduction of gold ions [13] is used to produce AuNPs [1,2,13,14], which are then surface functionalized [15,16] and increasingly either injected or implanted into rodent preclinical models for a specific assessment (e.g., imaging, targeted drug delivery) [17–19]. Other NPs have their characteristic processing variables. Biotechnology applications including in vitro diagnostics [1,20] and in vivo delivery [17b] should also abide by a standard NP validation process. Key variables that need to be carefully assessed, controlled and characterized during this biomedical application process are shown. Table 1 provides a more complete listing of the characterization needs for such NP studies targeting biotechnology applications.

3. Nanomaterials Biomedical Applications often Equate to Nanoparticles

Current biomaterials interfaces and surfaces in biotechnology and medicine are the product of enormous efforts focused on fabricating, controlling, and characterizing materials interactions in physiological fluids, and with tissues and organs. Critical biological and physiological components that interact with nanosystems include thousands of host proteins, coagulation pathways, cell receptors and membrane components, immune activation systems, filtration organs, intracellular enzymes and redox pathways, microtubule and trabecular systems, genetic materials, and microbial endo- and exo-toxins. Specialized protocols and surveillance methods (e.g., as embodied in ISO and ASTM specifications and FDA QSR regulations) have been validated for medical device, drug, and biomaterials development and testing. This has been accompanied by the evolution of tools to provide increasingly sophiscated amounts of high-density information on bio-surface behaviors at greater resolution. Nonetheless, surface analytical tools capable of *nanoscopic details* are still limited, even under the best UHV conditions.[21] Most of these tools do not have the spatial resolution to examine an individual NP; all results are thus an average value from ensembles of NPs and background in spaces beneath and around them, and from bulk material in multilayers. Also, the high-energy probes (e.g., X-rays, electrons, and ions) used in many surface analysis techniques can cause changes and degradation of NPs being analyzed (reduction of surface oxide layers, change in particle shape and size, etc.). Critical surface analytical information on nanotechnologies and nanomaterials lags substantially behind that obtained for identical materials and systems at larger, macro- and micro-scopic scales. The high surface curvatures of small NPs also present special challenges to interpreting the few surface analysis characterization experiments now performed. No methods are available to produce much meaningful information reliably on nanophases in biological milieu – even light scattering studies on nanosystems can be plagued with fundamental interpretive issues.[4] When nanobiomaterials are dimensionally similar to proteins, characterization of various interactions is exceedingly difficult, especially in milieu where relative mass fractions of proteins greatly exceed that of the materials.

Given the enormous contributions of surface science to modern biomaterials science and engineering, the current lack of careful surface science studies and rigorous analyses on nanosystems deployed in biological systems produces a position of relative ignorance that creates problems in trouble-shooting and improving performance in biomedical systems. The recent U.S. FDA report on nanotechnology[22] underscores this concern with regard to new safety and efficacy assessments required to approve nanotechnology for human use.[23] A recent review substantiates the correlation between surface chemistry in nanomaterials and their reported toxicity.[24] Additionally and distinctly, it is likely that nanophases present the same surface heterogeneity, contamination, and surface stability problems as their macrophase analogues (why not?), particularly when surface modification is frequently attempted on most NP systems for biological applications. Challenges for controlling and characterizing nano-phase materials with significantly enhanced surface areas and reactivities are daunting, but it is essential for the responsible scientist to address them expediently to best exploit the unique features of nanoscale properties and materials.

Significant to the confounding nano-surface scenario, consider the intrinsic reliance of nanomaterials and colloids on (1) surface-bound stabilizers to prevent aggregation, and (2) fabrication additives required for nanomaterials synthesis. These include surface-active products of dispersal, etching, forming or lithographic techniques used in nanofabrication.

For example gold NPs are routinely prepared via reaction of gold chloride with sodium citrate,[4,13] producing citrate- and chloride- covered gold NPs, stabilized from immediate aggregation. However, further functionalization of the gold NPs (e.g., with alkyl thiols[15,16]) can result in unwanted aggregation. Other methods are now employed to produce in situ coated gold NPs, stabilized as they form in reducing conditions in liquids.[14,25] Nanomaterials processed in these surface active agents, additives and stabilizers are routinely used in biological and biomedical applications without much of the "normal" accompanying information on their surface properties traditional to the biomaterials field. For example, published data for XPS, surface enhanced Raman spectroscopy (SERS), or time-of-flight secondary ion mass spectrometry (ToF-SIMS) from NP chemistries commonly used in biomedical and biotechnology applications are difficult to find.[26–31] Enormous, highly reactive NP surface areas imply a need for vigilance against their significant potential for contamination potential: from ubiquitous surface-active microbial endotoxins, adventitious adlayers, mixed oxides, ionic adsorption/exchange, enhanced corrosion products and Ostwald ripening and coarsening. *All of these surface modifications, intentional or not, can have enormous effects on their properties in biological systems!*

The issues surrounding NP stabilization are currently under much discussion.[4] A very recent laser Doppler velocimetry electrophoretic mobility study on Au, Ag, Pt, PbSe, and other metallic nanoclusters concluded that these phases do not exhibit the high net surface charge naively expected for charge stabilization by DLVO theory. Surface charge for the systems studied ranged only within –2, –1, 0, or +1 (i.e., cationic +1 for a $PbSe/RCO_2H$ system), and was altered by the surfactants/ligands selected and amounts used.[32] Hence, relatively small net charges seemingly impart considerable DLVO-type stability, but perhaps not adequate for reliable stabilization in physiological milieu (i.e., 0.14 M salt and >50 mg mL^{-1} soluble proteins). Alternatively, NPs well-stabilized "as prepared" may become very unstable (or more stable) as a function of their changing surface ligation in biological milieu. *In fact, definitive studies of NP stability and aggregation in physiologically relevant milieu are difficult to find*. A recent study shows significant degradation of alkyl thiol capped gold nanoparticles can occur in the presence of halide ions and oxygen.[33] Gold NPs are interesting in this regard since they are increasingly applied in biotechnology in different architectures with various properties and performances.[2,17,20,34,35] Historically, the study of colloidal gold properties and stability in water originated over 150 years ago[36] with the formal science of gold colloid stability first studied just over a century ago.[37] Gold colloids remain the most reported nano-system to date: as such, gold NPs and nano-materials would not seem to be that novel unless unique compositions might provide significantly new therapeutic or technological properties. Moreover, despite increasing in vivo use, the actual scientific basis for understanding fundamental interactions of gold and other NPs with complex (in vivo) full organismal and physiological systems remains largely uncharacterized. While the general consensus is that gold should remain unreactive and therefore as safe as any nanomaterial in vivo, some data are emerging to suggest otherwise.[17b]

4. Carbon-Based Nanomaterials Considerations

As another example, carbon-based nanotubes and C60 "buckyballs" are receiving increased attention for applications in vivo[38–40] and associated toxicity[41,42] These materials are well-known to actively adsorb volatile polycyclic aromatic hydrocarbons (PAHs) co-produced as part of the carbon nanophase manufacturing process.[43,44] As a model study, aromatic pi-pi interactions for pyrene adsorbed on graphite (HOPG) models estimate such adsorbate interactions at ~10 kcal mol^{-1}.[45] PAHs raise concern since they are known carcinogens.[46] Risks of PAH release from such high specific surface area carbon materials (i.e., up to 1000 $m^2\ g^{-1}$; 1 microgram of single wall nanotubes might plausibly release 10–100 attomoles of PAH) into biological systems is a confounding intrinsic toxicity issue distinct from that of the nanophase materials themselves (and perhaps higher risk). PAH surface 'contamination' also raises concern about surface modification and derivatizations. Functionalization of these NP surfaces might also involve unintended reactions with adsorbed PAHs, not the target carbon nanomaterial surface, a situation difficult to interrogate or prove. Rarely are surface cleaning or sensitive characterization protocols described for these carbon nanophase materials before and after surface chemistry and derivatization and exploitation of these products for biotechnology or biomedical use. Hence, biomedical use of these nanophases with adsorbed PAH adlayers from processing is likely common, and biological impacts of this 'contamination' remain to be reported. Despite new assertions that carbon nanotubes safely deliver drugs in vivo with no observed side effects,[38] and also escape host filtration organ scavenging,[47,48] other studies seem to contradict this.[49,50] Nonetheless, most studies are without any surface analysis of the dispersed nanotube phase deployed in vivo. What and how many contaminating molecular entities are actually delivered from these high surface area materials (both deliberate and inadvertent) and the stability of their modifications in biological milieu are largely unknown. Factors contributing to different circulation times and clearance rates cannot be ascertained. Comparisons of these increasingly important carbon-based materials across different preparations are practically impossible without such reporting and careful analysis.

5. Nanomaterials Surface Considerations when Deployed in vivo

Technology, not science, appears to be a primary impetus for the rapid implementation of nanomaterials in complex in vitro and in vivo biological operating environments where lit-

tle systematic science is reported, let alone dissected and understood. Bioavailability, pharmacokinetic and biodistribution analyses required for pharmacological understanding of NPs from nearly all chemistries in medicinal or biomedical technologies are scant.[51] Most literature reporting NPs in vivo administration dose substantial quantities (i.e., mg kg^{-1}) simply to detect any effects and expected particle biodistributions, but not the *unexpected fraction found in unintended sites* (i.e., in filtration organs, microvasculature, remote emboli, aggregated masses, and phagocyte scavenged). Admittedly, these are difficult studies both to perform and then interpret – *finding NPs in vivo remains very challenging*. Furthermore when these requisite biodistribution data are not readily available, or when NP behavior in simple in vitro models does not reflect in vivo behavior,[52] interpreting toxicity or therapeutic cause-and-effect is largely an empirical exercise. Initial, albeit sometimes conflicting, correlations of toxicity with NP surface area and surface chemistry are emerging and remain to be explored and explained.[24,53,54] Fundamental NP properties and distributions in biological systems remain largely unknown, and the data published are frequently contradictory or inconsistent, also when few common materials and methods are compared. Some very intriguing properties have been reported (e.g., selective passive deposition in disease sites, spontaneous killing of bacteria, ability to traverse the blood-brain-barrier, avoidance of immune cell activation, cell membrane penetration without endosomal processing) that remain to be validated. Significant concern has also been expressed about toxicology[55] but with little consensus on assay methods or in vivo safety.[24] In fact, as a testament to the strong interest in this emerging nanotoxicology debate, among the most downloaded papers from *Toxicological Science* since 2004 are two acute, sub-chronic studies of pulmonary toxicity of carbon nanotubes in rodent models.[56,57] More recent follow-on reports consistently support an unusual pulmonary inflammatory response to single wall carbon nanotubes, accompanied by early fibrosis and oxidative stress.[58] A recent addition to this complexity surrounds the observation that tissue distributions and circulating lifetimes for nanobiomaterials in systemic circulation appears to be influenced by particle morphology.[59] Clearly, the nanotechnology field is far from an informed consensus about in vivo safety and efficacy mechanisms – prior to mid 2005, fewer than 10 publications could be found on this topic.[60] Proper scientific attention to these critical nanomaterial surface characterization issues provides one important way *to improve NP science in biomedical applications*, full understanding of their biological response and strategies for appropriate exploitation as technology in the health sciences.

6. Polydimethylsiloxanes as Ubiquitous Uncontrolled Surface Contaminants

A related topic directly relevant to the puzzling interfacial scenario for nanomaterials concerns the unique interfacial and surface-active properties of polydimethylsiloxane (PDMS, silicones), ubiquitous in microfabrication as a polish, lubricant, mold release, bulking agent, elastomeric and adhesive additives, and pump oil. The surface properties of PDMS and silicones as contaminants have been studied in biomaterials[61] and other materials contexts for several decades.[62–68] Publications involving PDMS and microfluidics, nanotechnology, surface patterning and soft lithography, and aspects of biotechnology have increased exponentially since 1990. Now, PDMS finds increasing application in sub-micrometer patterning, microfluidics, and MEMS devices;[69] biotechnology routinely employs in complex biological milieu. Interestingly, publications involving PDMS and surface analysis in these miniaturized applications have increased far less dramatically. Yet, ready transfer of PDMS oligomer residue from soft lithographic stamps to patterned surfaces is well-known,[67] as is the ubiquitous nature of volatile, surface-active PDMS in common pump oils, glassware greases, vacuum systems and lab environments, and even cleanrooms.[70] *PDMS ranks next to the plasticizer dioctylphthalate as the most commonly identified adventitious surface contaminant observed on surfaces under high-resolution surface analysis.*[71] Methods and metrics for PDMS surface contamination are well-known and accessible. Yet, little has been reported about adventitious PDMS contamination on numerous nanobiotechnology systems involving its routine direct (i.e., soft lithography, micro-contact printing, molding and stamping) or indirect (routine synthesis and laboratory contamination) use.

The potential for ubiquitous PDMS surface contamination to alter virtually every known and reported biological response is immense – from deliberate cell-adhesion peptide immobilization to protein adsorption to cell and bacterial adhesion, immune response, fluidics and wetting processes, biopattern affinity fidelity, molecular imprinting, surface capture bio-assay, and lithographic transfer. Nanopatterned metallic and organic features, lines, islands and structures fabricated from PDMS-facilitated templating might actually present little true surface chemistry intended to the external biological environment (i.e., the high surface energy metal or polymer is covered with an overlayer of the mobile low energy PDMS residue), but this likelihood as not yet been actively considered in current data interpretation. Additionally, PDMS is known to be highly reactive with soluble proteins and surfactants found in biological milieu, can exhibit negative zeta potentials in aqueous systems, and can be made water-soluble by plasma or oxidative processes. Non-specific PDMS transfer and surface adsorption processes and resulting biological response to fabricated nanostructures might therefore have much more to do with surface contamination and little really to do with lithographed or patterned structure surface chemistry. For example, fluorescently labeled proteins patterned onto a PDMA-stamped surface can be observed fluorescently but no protein signals are observed by surface-sensitive secondary ion mass spectrometry (ToF-SIMS) since the entire surface is covered with PDMS; *protein fluorescence emits through this PDMS overlayer*. It is therefore likely that many

nanofabricated particle and patterned systems are highly surface-contaminated with PDMS, confounding structure-property relationships. With numerous precedents for interfacial reactivity in biological systems, PDMS warrants a renewed attention from the surface science community for its impact on nanomaterials properties in diverse current applications. Little attention has been directed to date on these significant contamination issues on high specific-surface area nanomaterials.

7. Nanosurface Analysis is Required

It is readily apparent that few studies to date actually report true versus inferred nanomaterials surface chemistry or attempt the rigorous, tedious analysis required to make a valid judgment about the nano-surface chemistry involved in most biomedical applications. This problem surrounds many other potential surface contaminants in nanophased systems, especially those added ubiquitously to stabilize these systems in aqueous milieu. Continuing efforts to impart specific nanoparticle surface chemistries, displace contaminants, control the dispersed properties and surfaces in ionic or biological media, and bioconjugation or bio-immobilization capabilities are often poorly characterized. Following modification of the material surface, the putative assumption that gross physicochemical characterizations at macro-scales may be reliably extrapolated to the micro- and nano-scale is likely often erroneous. The seminal physicochemical characterization parameters applied to macro-scale materials, such as yields, coverage, heterogeneity, consistency, repeatability, stability, and metrics are not commonplace in the nanomaterial community and alarmingly, more often ignored in nanobiotechnology reports. In addition, the intrinsically high surface curvature for the smallest NPs certainly affects the quality of any overlayer deposited or adsorbed onto these materials. For example, it is highly unlikely that alkyl thiols form the same well-ordered, dense monolayers with minimal defects on 5-nm gold NPs as on flat, macroscopic gold surfaces. Figure 4 shows schematic cross-sections of alkane thiol molecules assembled onto flat and NP surfaces. The flat gold surfaces typically have a <111> orientation and the sulfur atoms of the alkane thiols will chemisorb into the 3-fold hollow sites producing a well-ordered SAM with a chain tilt of 30–35 degrees from the surface normal. Depending on the NP shape both <111> and <100> terrace planes will be present (see Fig. 2). The atoms in these two low-index planes have different coordinate numbers and atomic spacings, affecting the packing density and lateral ordering in the SAMs. In addition the edges between the NP terraces will act as defect sites where the alkane thiol molecules can disorder (see Fig. 4b). In this sense, the nano-bio-community might often be 'running blind' with high surface area materials applied in complex systems, and erroneously predicating observed results on unproven presumptions about surface chemistry. This practice presents a substantial opportunity to improve the science of nanomaterials and significantly impact

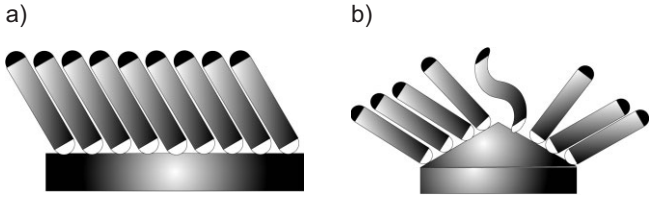

Figure 4. Schematic cross-sections of self-assembled alkane thiol molecules on a flat (a) and nanoparticle (b) gold surfaces. On the flat gold surfaces, which typically have a <111> orientation, the thiol groups (white) can chemisorb into the gold substrate 3-fold hollow sites allowing the adlayer alkane chains (grey) to pack laterally into a well-ordered monolayer with a chain tilt of 30–35 degrees from the surface normal [16]. This provides a spontaneous uniform arrangement of adlayer terminal groups (black) at the outermost surface of the SAM. These terminal groups are readily varied across a wide variety of chemistries, providing versatile opportunities to stabilize particles and impart specific surface properties. On the gold nanoparticle surfaces, even if the alkane thiol molecules form a well-ordered SAM on the terraces, the edges between terraces will act as defect sites where disclinations and disorder of the alkane thiol molecules occur.

the understanding of mechanisms of such materials performance in biotechnology.

To rephrase these assertions, the remarkable results often proclaimed for unique nanomaterials applied in bioanalysis, biotechnology, biomaterials, tissue engineering, catalysis, molecular imprinting, and (micro)fluidized systems, need to be held to the same rigorous scientific and characterization standards that the more mature, traditional macro-biomaterials community has come to expect for decades. Classical colloid characterization tools are only marginally helpful to date: a very recent, thorough review underscore the deficiencies of many of the more-used analytical methods and their pitfalls when applied to nano-phase materials.[4] Little real surface science has yet been performed to equivalent standards on nanosystems in biomedical applications compared to macroscale biomaterials, or even the more mature catalysis, microelectronics and electrochemical fields. The nanobiotechnology field, for credibility, safety, efficacy and maintenance of good science, needs to demand equity and rigor to improve scientific approach and conduct on these systems. The properties attributed to high specific-surface area, reactive and metastable materials systems require rigor simply to understand structure-property mechanistic features important to interpreting results. Certainly, since NPs of various chemistries are the nanotechnologies targeted for nearest-term commercialization in various products, future GMP certification and FDA filings will require materials quality controls. This necessary oversight includes careful certification of surface composition, protection from contamination, stability and toxicity behaviors related to safety and efficacy in therapeutic and medical applications. For this to be accomplished, nanomaterials surface analytical methods need to be better developed to improve nanosurface scientific capabilities for characterizing biomedically interesting properties. Additionally, standardized surface characterization routines, methods and analytical benchmarks, and quality management systems should be insti-

tuted for high specific surface area materials to allow facile comparisons of nanophase materials properties and behaviors.

The nanobiotechnology community might best re-consider the limitations of current analytical and synthetic capabilities, current physicochemical understanding, and especially the *realistic* control of nano-phase materials for the desired application(s). Surface scientists have laid extensive groundwork of seminal analytical standards and expectations to properly correlate the structural and functional relationships in numerous macro-scale biomaterials and also precedent non-biomedical nanoparticle systems. In fact, nanophase surface structure, adsorbate reaction complexity and its mechanistic ambiguity are evident in even the 'cleanest' (i.e., high-vacuum) NP-based heterogeneous catalysis studies. Given an extensive 40+ years of research in macro-scale complexities and methods development in biomedical materials, and associated standard operating protocols in obtaining marketing approvals with regulatory agencies, perhaps today's scientists and engineers are improperly equipped, guided, and trained to adequately assess nano-phase physicochemical properties and the concomitant impact on their in vivo physiology and toxicology. As such, nano-scale surface characterization of nanobiomaterials appears to be a 'sleeping giant' that must be carefully activated and effectively harnessed. This future technology area is capable of providing innumerable new interdisciplinary scientific and engineering opportunities to rigorously produce a confident biotechnology template for nanosystems similar to that of more traditional surface science. A cursory overview of these potentially fruitful research areas include attention to consistent and standardized surface properties, reactivities and aggregation phenomena in biological milieu, levels of adventitious contamination, natures of adsorbates and stabilizers, bio-interfacial behavior, and complete pharmacological assessments necessary for in vivo applications. Importantly, new analytical tools and reliable processes are required for many of these capabilities to become widely adopted and routine.

A summary of analytical recommendations to address challenges common to synthesis, functionalization and biological applications of nanomaterials as a first objective is provided in Table 1. Additionally, as example sample cases, several recent nanomaterials publications are highlighted to provide some recommendations about implementing comprehensive assessment for nanosystems deployed in vivo. First, commercial gold NP preparations of several forms have recently been used in small animal solid tumor models where NPs are injected in vivo, allowed to find the solid tumor mass and then subjected to external localized radiation to eradicate the tumor.[18,19] Commercial nanomaterials should come with some minimal quality analysis validation, and specifically and importantly for in vivo applications, should be certified for endotoxin contamination (i.e., distinct from sterility), and levels of polydispersity, surface-active agents, and other relevant biomaterials properties. Despite both cited gold NP publications reporting extensive in vivo NP properties, neither study provides this NP characterization information, nor any materials analysis important to understanding host biodistribution or therapeutic responses observed, and reasons for possible adverse events. Additionally, a frequently stated observation in the absence of quality toxicological assessments is one of "apparent" safety and efficacy – that is, if the animal did not die of an apparent adverse event, then the material is deemed biocompatible. There are obvious limitations to such an interpretation.

The Table 1 checklist would suggest that certain minimal materials properties be assessed and reported for biotechnology, including dry-phase NP purity and surface analysis, followed by endotoxin screening, and stability and aggregation assessments using light scattering and/or centrifugation sedimentation assays in protein-containing milieu. Frank assess-

Table 1. Recommended characterization checklist for the synthesis, functionalization and biomedical applications of nanoparticles.

1) Researchers synthesizing and functionalizing nanoparticles should perform complete physical and chemical characterization.
   - Determine shape, size and size distributions of nanoparticles in relevant milieu
   - Determine elemental composition and molecular structure of the nanoparticles and any functionalized overlayers
   - Identify and quantify the presence of any surface contaminates introduced during the synthesis and fabrication steps
   - Quantify the extent of reaction and occurrence of side reactions during functionalization
   - If applicable, characterize the electronic and optical properties of the nanoparticles
2) Researchers using nanoparticles in biological environments should carefully assess interactions of nanoparticles with the biological environment.
   - Quantify the reactions of relevant species in the biological environment (i.e., biopolymers, cellular elements, enzymes) with the nanoparticle surface, including both specific and non-specific deposition of biomolecules (proteins, lipids, etc.)
   - Assess nanophase dispersed stability under biological conditions and over time.
   - Monitor the transport of nanoparticles within cells under cell culture in serum-containing culture media.
   - Monitor biodistribution in vivo (percent actually delivered to the target cells or tissue, percent delivered to other cells and organs, pathways for elimination from the body, etc.)
   - Assess toxicology and pharmacology with standard protocols used in drug screening and histology, both in vitro and in vivo.
   - Assay nanomaterials and wash buffers for presence of endotoxin contamination.
3) Researchers characterizing nanoparticles should develop new techniques and analytical protocols to produce new data relevant to biotechnology applications.
   - Known limitations of translating in vitro techniques to in vivo behavior should be recognized and consistently improved with innovation and technique development.
   - New techniques and protocols need to provide information about the physical and chemical states of nanoparticles in vitro and in vivo in complex physiological milieu.
   - New techniques and protocols should limit degradation and changes in the nanoparticles under analysis.
   - Ideally, new techniques and protocols should be capable of analyzing individual nanoparticles rapidly and collating these data to produce ensemble averages and statistics.

ment of NP coating stability under in vivo conditions and its possibility of sloughing or leaching[72] is important. A recent polymer surface-modified gold NP strategy used for in vivo imaging[73] and another in vivo approach using organically modified silica nanoparticles for gene delivery to the brain[74] also typify some problems of characterizing and comparing the many customized 'home-brew' nanomaterials now finding their way into pre-clinical in vivo testing. Both of these studies modify NP surface properties with organically coupled adlayers. The PEG-modified gold NPs are assayed for coating stability in vitro using a serum aggregation assay (visible observation endpoint) that is relatively insensitive and non-quantitative, but without a lot of analytical options for such testing since light scattering methods are complicated by serum protein presence. The silica NP study cites previous XPS analysis of this organically modified surface, and filter-sterilization in buffer. Neither assays for adsorbed endotoxin or surface contamination known to be ubiquitous in laboratory preparations. Both preparations are injected directly into rodents from buffers with no known effects of how these formulations behave in tissue or blood (e.g., aggregation that could affect organ filtration and lesion extravasation, and host immunopotentiation). Indirect analysis (i.e., local tissue imaging or gene expression) is used as evidence of successful NP delivery for intended in vivo functions, showing promising results. Histological analyses beyond the immediate desired local tissue effects are not reported.

In the second case study, blood-borne single wall carbon nanotube (SWNT) distribution in vivo was studied using attached radiotracers[49] or intrinsic near infrared fluorescence.[72] In the former case, phospholipid-conjugated polyethylene glycol (PEG) molecules were applied to SWNT surfaces by non-covalent adsorption and shown, using directly observed turbidity measurements, not to aggregate upon heating in buffer nor when mixed into serum. Limitations of such stability measurements 'done by eye' are in sensitivity: nephelometry or light-scattering measurements are definitive in buffer. What stability data are possible using optical methods in serum with such finely dispersed, dilute particle systems remains to be determined. As the authors used sensitive ^{64}Cu as a chelated radiolabel to the PEG chain, SWNT surface analysis could have provided an estimate for radio-isotope surface loading efficiency of the ligands, lipid-PEG coverage, stability and turn-over. Radiometric assay surveys only the chelated label but in tandem with XPS or ToF-SIMS of non-radioactive analogues provide substantially more information. The second SWNT study[72] claims using various spectroscopies (i.e., Raman and fluorescence) that SWNT rapidly lose their PEG passive coating in serum. That this was assessed directly is admirable and results in a logical claim given no known specific binding affinity between SWNT chemistry and PEG, and the known surfactancy intrinsic to many components of serum. The documented PAH and other adsorbate content common to SWNT surfaces (vida infra) begs the question as to the true bound state of other added NP and SWNT stabilizer coatings, as well as the leachates in vivo, and a precise chemical meaning for 'pristine SWNT'.[72] Both covalent and non-covalent stabilizer coatings should be subject to more rigorous, definitive surface analysis to assess many of their qualities in relevant biological conditions, as has been done historically and extensively in biomedical surface-modified devices. This would include standardizing rigorous, defined surface cleaning and purity conditions, aggregation, coating stability, and opsonization and cytotoxicity assays using serum proteins and various cell models. Such information would be used to determine physical states in vivo and correlate inputs to understanding pre-clinical systemic toxicological assessments and pharmacodynamics.[42,49,50]

8. Conclusions

In summary, nanobiomaterials present new challenges for assessments of biostability, biocompatibility, pharmacology and biodistribution. Many nanosystems promoted for biotechnology or biomedical applications in vitro and in vivo remain to be characterized to standards typical for conventional clinically approved biomaterials. NPs represent the current operational "poster-child" for nanotechnology – now often reported in many forms and materials, and blithely advocated for many biomedical and biotechnology uses. Nonetheless, nanotechnology remains scientifically premature in the ways described above for exploitation in many biomedical applications, *particularly those in vivo*. Particle analysis is difficult enough in vacuum, let alone in physiology. Commonly applied electron microscopy methods have plausible connections to reality for analysis of gas-phase NP applications. This utility is not so apparent for NPs in physiological milieu, but few other characterization methods that relate to physiological media are currently available. Since NP surface area is a predominant biointerfacial property of NP systems, surface analytical rigor is critical. Mechanistic connections from nano-phase surface properties to biocompatibility, pharmacology and toxicology continue to suffer from lack of appropriate methods and controls, and lack of published reliable surface data. Subsequently, in vivo nanotechnology applications are generally treated as a 'black box' where structure-property relationships cannot be scientifically validated due to intrinsic complexity of these systems and the lack of rigor required to extract new information. As a result, much empirical and correlative 'science' is reported: many reports on similar systems (i.e., gold or carbon nanotubes, among many other home-brew nanomaterials) are contradictory or conducted under conditions that preclude accurate comparisons across seemingly similar systems. This is a dangerous proposition for biomedical validation, requiring contingency plans to push ahead with more rigorous scientific studies on these difficult nanosystems. This effort will require innovation, persistence, rigor and an appreciation for the dual complexity of these two challenging systems interacting. In fact, this process parallels surface science innovation strategies of the past applied to other difficult technologies, including microelectronics, catalysis, bio-

medical devices, and 'wet systems' immersed in liquids. Nanoscience and nanobiotechnology should together be co-developed as a hand-in-glove for biomedical applications. This coordination would ensure in this case, as in past surface science and catalysis work, that the immense surface properties and unique reactivities intrinsic to nano-phase materials receive the proper attention from appropriate surface analytical chemistry in tandem with improved inventories of their desired properties and better understanding of their applications. This is best facilitated by encouraging and implementing nanomaterial analytical technique evolution and standardization with the goal of producing a complete and comprehensive understanding of nanomaterial surface properties that yield reliably the desired, novel biomedical properties best achieved through size reduction.

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