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Engineered nanoparticle bio-conjugates toxicity screening: The xCELLigence cells viability impact

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Abstract

Introduction: The vast diverse products and applications of engineered nanoparticle bio-conjugates (ENPBCs) are increasing, and thus flooding the-markets. However, the data to support risk estimates of ENPBC are limited. While it is important to assess the potential benefits, acceptability and uptake, it is equally important to understand where ENPBCs safety is and how to expand and affirm consumer security concerns.

Methods: Online articles were extracted from 2013 to 2016 that pragmatically used xCELLigence real-time cell analysis (RTCA) technology to describe the *in-vitro* toxicity of ENPBCs. The xCELLigence is a +noninvasive *in vitro* toxicity monitoring process that mimics exact continuous cellular bio-responses in real-time settings. On the other hand, articles were also extracted from 2008 to 2016 describing the *in vivo* animal models toxicity of ENPBCs with regards to safety outcomes.

Results: Out of 32 of the 121 (26.4%) articles identified from the literature, 23 (71.9%) met the *in-vitro* xCELLigence and 9(28.1%) complied with the *in vivo* animal model toxicity inclusion criteria. Of the 23 articles, 4 of them (17.4%) had no size estimation of ENPBCs. The xCELLigence technology provided information on cell interactions, viability, and proliferation process. Eighty-three (19/23) of the *in vitro* xCELLigence technology studies described ENPBCs as nontoxic or partially nontoxic materials. The *in vivo* animal model provided further toxicity information where 1(1/9) of the *in vivo* animal model studies indicated potential animal toxicity while the remaining results recommended ENPPCs as potential candidates for drug therapy though with limited information on toxicity.

Conclusion: The results showed that the bioimpacts of ENPBCs either at the *in vitro* or at *in vivo* animal model levels are still limited due to insufficient information and data. To keep pace with ENPBCs biomedical products and applications, *in vitro*, *in vivo* assays, clinical trials and long-term impacts are needed to validate their usability and uptake. Besides, more real-time ENPBCs-cell impact analyses using xCELLigence are needed to provide significant data and information for further *in vivo* testing.
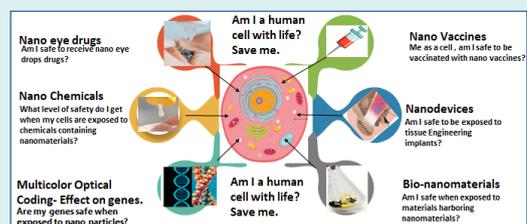
Introduction

The evolution of engineered nanoparticle bio-conjugates (ENPBC) has led to a wide spectrum of biomedical and environmental products and applications.[1-5] Ideally, ENPBCs are the formulation of any pharmacological active nanomaterial (e.g., nano-bio-conjugated, recombinant proteins, vaccines, nucleic acids, or bio-products)' that enable site-specific-targets of organs/cells with controlled release or to treat and prevent diseases (e.g., cancers, infections, bone tissue engineering, etc) in human.[4,5] In this context, nanoparticles (NPs) including those of gold, zinc, copper, platinum, carbon nanotubes and other metal oxides are widely used with varying distinctive therapeutic functions/properties.[1,6-8] The array of applications is based on NPs vast physiochemical broad multi-faceted properties derived from their nanosize scales and interactions.[1,4] However, the understanding of the diverse behavior of ENPBCs and characteristics within human cells is still limited. Therefore, simple, fast, and reliable *in vitro* screening techniques that detect cellular

toxicity effects are vital before they reach the environment and the market.

The ENPBCs *in vitro* or *in vivo* demonstrations have shown varying biological effects in animal cell development and reproduction.[9] However, the biological effects of ENPBCs concerning the human health have not fully been established as is the case with other commercially available pharmacologic active non-nanomaterials and antibiotics.[3,10-13] For example, commercially available drugs have written mechanisms of action, toxicity, and side effects whereas such information to date are limited for ENPBCs. This is because most nanodrugs are still at the developmental phase with limited safety trials. Addressing the undesirable effects of ENPBC require clear regulations that govern their toxicity in human and environment. Specifically, this requires a considerable amount of data and evidence-based information to support ENPBCs toxicity effect/impact, safety, tolerability, adverse reactions, and others. It may also include information and background on the toxicity profile and testing tools that could be used in the standardization and expansion of local, national, regional, and international regulatory frameworks for ENPBCs. Unfortunately, the non-availability of tools for estimating nanomaterials toxicity for standardization is one of the major setbacks regarding their regulations.

It must be noted that ENPBCs have a large surface area and volume ratio leading to a high diverse-level of interaction with assays, binding and bonding to chemicals, and materials thus giving false toxicity results.[11,14] For example, graphene families of nanomaterials can interact with assays by binding to lactate dehydrogenase (LDH) thus giving false negative toxicity results.[11] The use of xCELLigence real-time cell analysis (RTCA) technology system, in this case, is vital because it has some promising characteristics that limit ENPBCs interactions with various dye, enzymes and chemicals used in estimating cytotoxicity thereby reducing false-negative and false-positive outcome. In other words, xCELLigence *in vitro* toxicity testing provides real-time tissue cells' observation, cell growth, reproduction, and morphological effects without the interferences of non-external cells, chemicals and dyes interactions as in other conventional cytotoxicity testings.[14-17]

This study, therefore, describes ENPBC toxicity using the xCELLigence technology system and the role of regulatory bodies regarding ENPBCs and safety guidelines.

According to reports by Ke et al *in vitro* toxicity studies determined via xCELLigence instrument do not use external reagents that may confound the screening. In principle, xCELLigence toxicity testing is a noninvasive electrical impedance technique that continuously monitors and quantifies cell proliferation/viability, morphological changes and attachment.[12-14] The following steps describe a simple synopsis of the xCELLigence ENPBCs toxicity screening according to Hamidi et al (Fig. 1).[18]

It must be noted that all steps are conducted under strict
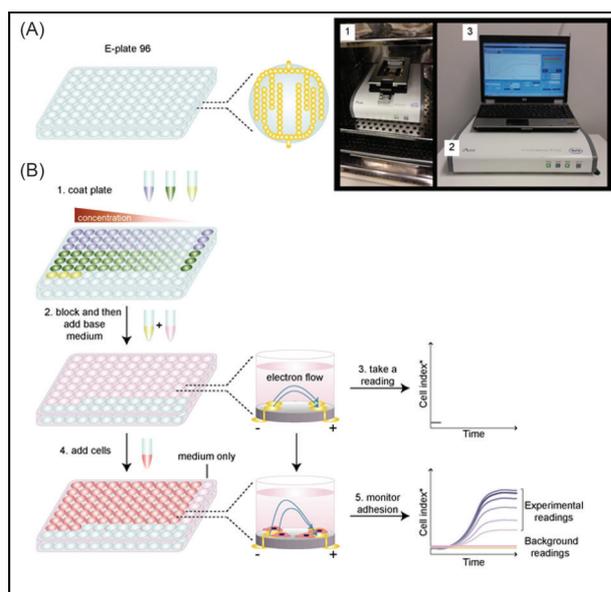
Fig. 1. xCELLigence protocol flow system: Adapted from Hamidi et al. Using the xCELLigence RTCA system to monitor cell adhesion.

sterile and instructional conditions. For further details, readers are referred to Hamidi et al protocol. Step 1: Set the startup xCELLigence RTCA program as instructed for use including the experimental layout, page layout, and the schedule page layout. Step 2: Prepare the test run cell adhesion experiment using the xCELLigence RTCA background reading. Step 3: Prepared study target cells are adhered to or seeded to the microtiter 96 well plates. The wells will be placed on the gold microelectrodes that impede the flow of the electric current between electrodes. Step 4: The adhered cells are seeded with the required growth tissue culture media in question. Step 5: Addition of various sterile concentrations of the ENPBCs without any external reagents to the tissue culture media. Step 6: Monitoring of the ENPBCs and tissue culture xCELLigence real-time viability profile from a humidified CO_2 incubator using an external computer system.

As for the standardization and regulation of nanomaterials, it must be noted that the toxicity levels depend on the type of ENPBC, the size and shape specific, surface chemistry, roughness, hydrophilic/hydrophobic characteristics, zeta potential, solubility and surface coatings and conjugated ligands.[15-17] As discussed already, despite the evolution of nanomedicine, coupled with other vast applications and nano-products there are no clear human safety screening techniques either *in vitro* levels. At the moment, the Consumer Protection Act Right requires local, government, national, regional and international regulatory organizations to protect consumers by setting/stating standards as well as published safety regulatory/instructions, protocols, and warnings that might evolve from some nano-consumable products.[19] Based on this, the National Institute of Health (NIH) and Organization for Economic Co-operation and Development (OECD)

have released calls for the conceptualization of and development of safety regulations of bi-nanomaterials of biomedical devices, food, cosmetics, chemical and industrial systems.[10,19]

Methodology and Data Sources
Online toxicological search themes were used to screen ENPBCs studies from March 2013 to December 2016 and conglomerate findings that used xCELLigence technology to describe *in vitro* toxicity. Additionally, a review of ENPBCs articles from 2008 to 2016 describing the *in vivo* toxicity using animal models was carried out. The ENPBCs articles included were those of significant toxicological information. The searched themes and topics covered were (1) *ENPBCs xCELLigence cells' viability,* (2) *chemistry of ENPBC,* (3) the role of the international regulatory agencies in ENPBCs toxicity, and (4) challenges in regulating ENPBCs.

Search methods
A literature search was conducted to extract studies that address the *in vitro* cytotoxicity of ENPBCs using xCELLigence tools as well as *in vivo* animal models. The articles included were those published in the English language (limitation of search) and provided a clear and comprehensive description of ENPBCs *in vitro* toxicity using the xCELLigence technique and *in vivo* animal models testing. The key search words used for the xCELLigence techniques search and screening were *xCELLigence nanoparticles toxicity, xCELLigence and nanoparticles screening,* and *xCELLigence and nanoparticles cell viability screening*. The PubMed advance mesh search used, for example, was xCELLigence[All Fields] AND ("nanoparticles"[MeSH Terms] OR "nanoparticles"[All Fields]) AND ("toxicity"[Subheading] OR "toxicity"[All Fields]). The xCELLigence *in vitro* toxicity testing technique was used because it reduces and limits confounding toxicity outcomes when compared to other conventional testing methods that use chemicals, dyes, and reagents.

For the *in vivo* ENPBCs toxicity estimation and description, only published work indicating the use of laboratory animals such as mice/rat/mouse were eligible. The *In vivo* nanoparticles toxicity, and *In vivo* nanoparticles testing/screening were the searched main keywords. For example, the PubMed advance mesh used was (("in"[All Fields] AND "vivo"[All Fields]) OR "in vivo"[All Fields]) AND ("nanoparticles"[MeSH Terms] OR "nanoparticles"[All Fields]) AND testing[All Fields]. Articles that used non-primate animal species such as fish and other aquatic animals (limitation of the study) were excluded.

The following were the main online search databases: Cochrane Library, PubMed, MeSH PsycInfo, Scopus and Google Scholar, Embase, Web of Science, BMC, Plos|One and Global Health, ScienceDirect. The Prisma proxy flow checklist methodology was used to identify, screen, and review the articles[20] as described in Fig 2.

Selection of studies
Two independent reviewers (CSY and GSS) reviewed the relevant studies.

Selection and management of studies
Using the Microsoft Excel spreadsheet, CSY and GSS independently reviewed the extracted studies, screened
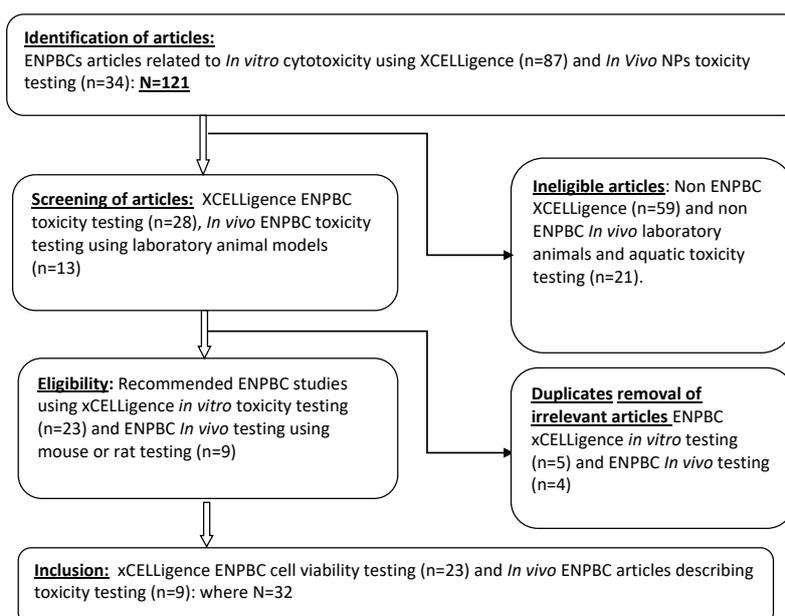
Fig. 2. The identification, selection and screening of ENPBCs XCELLigence *in vitro* viability and the *in vivo ENPBCs toxicity* articles flowchart for dose estimation.

the titles, and results. Thereafter, the pre-piloted checklist was applied to identify eligible studies. The eligible studies were further screened for duplicates and other irrelevant studies. To reduce discrepancies, a clear screening and selection approach was used based on the headings generated in Tables S1[14,21-43] and S2[24,28-31,38,44-51] (see Supplementary file 1). Similarly, some findings were further discussed to find a suitable outcome especially for the same studies extracted from conference abstracts and the full published articles.

Results and Discussion

The limited reliable and specific standardized protocols for determining the toxicity of nanostructures or nanosized bio-conjugate materials is still a challenge in both *in vitro* and *in vivo* bio-nanotechnology-applications.[1,16] Besides, the paucity of data has also compounded the difficulty of providing a generalized conclusion regarding the facts surrounding ENPBCs toxicities and regulatory standards. Out of the 121 articles, which were identified 23 (26.4%) of them, were used in this study are listed in Tables S1 and S2. They show 23 (71.9%) describing the xCELLigence-*in vitro* toxicity and 9 (28.1%) the *in vivo* toxicity of ENPBCs (Fig. 1). Of the 23 articles, 4 of them (17.4%) had no size estimation of ENPBCs, while the xCELLigence technology provided information on cell interactions, viability, and proliferation process. However, the xCELLigence cytotoxicity characteristics varied and were mostly due to the allotropic nature of the NP, dose, and size, as described in Table S1. For example, toxic ENPBCs were mostly those that initiated cell death, inflammation, disruption of cell membranes, DNA damage, and oxidative stress.[21-25] Non-cytotoxic ENPBCs were those that failed to initiate cell damage, had no cytotoxicity effect on the cells, were used for the diagnostic purposes/treatment, were biocompatible to cells, and induced no oxidative stress on the cells.[26-36] Partially nontoxic ENPBCs indicated both toxic and nontoxic effects due to size variations or the allotropic forms (e.g., carbon allotropes such as diamond, graphite, fullerenes, etc).[14,21,27-29,37]

The *in vivo* animal model provided further toxicity information where 3 out of 9 of the *in vivo* animal model studies indicated partial nontoxic animal effect, one was toxic while the remaining results recommended ENPBCs as a potential candidate for drug therapy with limited information on toxicity (Table S2). Some of the *in vivo* ENPBCs toxicity effects demonstrated programmed cell death, causing the release of H_2S.[29] The ENPBCs that exhibited *in vivo* nontoxic effect were mostly those that had the therapeutic effects of antibody-drug conjugate,[27] CTAB layers of gold for diagnosing rheumatoid arthritis,[30] surface coating polymeric NPs used for inhibiting cancerous cells[31,38-47] and felodipine-loaded polymers for pathological examination of different organs of Wister albino mice[50.] The ENPBCs with partial nontoxic effect were chitosan-coated polymers for drug delivery,[49] silver

NPs coated polymers that were found localized in various body organs,[48] and drug delivery polymeric NPs for anti-cancer.[51]

The findings indicated limited data and information regarding the regulation of ENPBCs toxicity. This may be due to limited available standardized protocols and methodologies for nanomaterials toxicities analyses. Similarly, there were no clear epidemiological studies or data from established longitudinal cohort studies describing the causal effect/impact of ENPBCs.

The *in vitro* xCELLigence cell viability test techniques were used as indicators for the first-line screening, observing cell growth responses and effects of ENPBCs on human cell cultures.[9,21-23] Similarly, the *in vitro* xCELLigence profiles were identified as broad sensitivity testing effects in cell cultures which were good precursors for specific toxicological testing in animal models and clinical trials in humans. Table S1 indicates and describes the diversity of the *in vitro* xCELLigence technique toxicity profiles of ENPBCs in cell cultures. The characteristics that were used in assessing the *in vitro* xCELLigence toxicity were: nature of ENPBC, shape, nanosize, animal cell lines used, dose toxicity estimation, and outcome effect on cell cultures.[52-55]

The toxicological, dose concentrations and effect of ENPBCs in cell lines showed varying toxicity levels. For example, citrate stabilized gold NPs (AuNPs) at a concentration of 1nM, 2nM and 5nM were shown to be moderately toxic to BEAS-2B cell lines.[14] Similarly, using Ru(II) complex (2–4) 4 at a concentration of 0.5nM, 1nM, 2nM and 5nM: the 0.5 μM was toxic to A549R cells resulting to the death of cells after a short interval, confirming the cytotoxicity of complex 4 against A549R cells.[23] According to reports by Li et al limitations to validate of xCELLigence technique impact are due to inadequate data or denominator indexes that could be used to quantify the behavior of ENPBCs in humans as well as the guideline monitoring methods and procedures for characterizing ENPBCs toxicity levels. Moreover, the limitations are also prevalent despite a host of other instruments including UV-Vis absorption spectroscopy, TEM, SEM, HrTEM, DLS, HPLC, ICP-MS, FTIR, and AFM for characterizing ENPBCs properties,[9] as listed in Table S1.[14,21-43]

The changes and mechanisms that take place in living cells in response to ENPBCs delivery are useful for understanding cell impact and after effect of ENPBCs.[17] The *in vitro* xCELLigence impendence technique has emerged as an alternative method that challenges the limited earlier traditional standards. The technique permeates cells to have direct contact with the ENPBCs without interference with other compounds or molecules as is the case with other *in vitro* testing methods.[11] For example, studies have shown ENPBCs interactions with MTT formazan assay crystals such as dyes like Neutral Red or Alamar Blue have resulted in conflicting

false outcomes.[44-45] Further, ENPBCs and lactate dehydrogenase (LDH) assay interaction has been found to confound outcome measurements, and thus, resulting in inconclusive endpoints results.[46] On the other hand, the *in vitro* xCELLigence technique incorporate an integrated sensitive impedance detection sensor chips system at the bottom of the cell culture plates that track ENPBC-cell interaction changes and their morphologies in cell and cell proliferation.[14,17] This method shows internalized ENPBCs within cells and their subsequent intracellular aggregation.[14] However, *in vitro* xCELLigence techniques seem to have some limitations as well. According to findings by Meindl et al the xCELLigence technique was found to be less sensitive to the CNT-induced cytotoxicity. The reasons for the less sensitive were not mentioned in the study. This indicates that xCELLigence techniques may not be a good standard for ENPBCs screening and thus, other *in vitro* cytotoxicity methods may be explored.

To enhance ENPBCs acceptability, the usage and sustained uptake in human drug delivery systems, the *in vivo* animal model toxicity testings are the next steps for assessing the activities, effect, and impact of ENBPCs in humans. Table S2 describes the *in vivo* toxicity effect of ENPBCs in animal models with varying effects from toxic to non-toxic ones. The varying effects occur at the absorption level, blood concentration, distribution and organ concentration, metabolic-breakdown and excretion/elimination phases.[47-48] The effects are influenced by several factors, depending on the nature of the ENPBC including size, the zeta potentials, shape, and the ENPBC general characteristics.[48] Other inherent influential characteristics are the route of administration, dose-effect, time of dose-to-cell effect interaction, ENPBC-cell–interaction, and host-immunological profiles.[48] For example, the intranasal inoculation of Fe_3O_4 magnetic nanoparticles (MNPs) coupled with polymer poly(lactic-co-glycolic acid) (PLGA) at a concentration of 50 μL containing one mg/mL of MNP was tolerated *in vivo* but inhibited lung adenocarcinoma growth in mice.[31] In another study conducted by Wang et al while performing aortic allografts with 20 nm hydrogen sulfide (H_2S) mesoporous silica NPs (MSNs) at the concentrations of 3.13 μg/mL to 800 μg/mL, the study revealed that the release of H_2S in a controlled fashion can result in apoptosis of graft endothelium. An experimental study by Recordati et al reported the mid-zonal hepatocellular necrosis, gall bladder and hemorrhage toxic effects when treating mice with an intravenous dose of 10 mg/kg of 10nm silver NPs (AgNPs) coupled with citrate (CT)/polyvinylpyrrolidone (PVP). These varying outcomes are described in Table S2.[24,28,30-31,38,44-51] Altogether, more *in vivo* animal predictive data are needed to validate standard methods for determining the safe applications of ENPBCs in humans.

Chemistry of engineered nanoparticles bio-conjugates
It should be recalled that ENPBCs have multifactorial complexity that may span from structural activities and interactions with biomolecules/cellular structures including proteins, membranes, cells, DNA, and organelles.[17,48] Some of these effects include the generation of radicals/oxidative stress, modulation of inflammatory profiles, and mutations.[1,9,52] It has also been found that interference of ENPBC with detector instrument, assays, dyes use in cytotoxicity (neutral red, Alamar blue, etc) and buffers have resulted in false negative/positives outcomes and misinterpretation or inappropriate toxicity results.[44,45] ENPBCs in this context are referred to as core nanoparticle and/ outer stabilizing layers made up of ligands (bio-conjugates). The bio-conjugates may be drugs, carboxyl (-COOH), amine (-NH_2), hydroxyl (-OH), methyl (-OCH_3), esters (-CO- OR), PEGylated layer intended for drug delivery applications. These bio-conjugates have great cellular interaction with cells, biodegradation as well as the elimination of the core nanoparticle. Therefore, elucidating toxicity effects that occur upon the interaction of functional groups with the cell surfaces or cellular components can lead to elevated intracellular reactive oxygen species resulting in DNA, lipids, proteins damage, cytotoxicity, apoptosis, unregulated cell signaling, tumor enhancer.[48,52-54]

Engineered nanoparticles bio-conjugates-structures
The nature/size of ENPBCs can be investigated using various optical systems, including TEM, SEM, ICP-MS to determine bio-distribution. Furthermore, the FT-IR can be used to determine the chemical bonding of the functionalized ligands and HR-TEM the capping image with ENPBCs.[53] The structure of ENPBCs can initiate an effect that may lead to bio-persistence, bio-durability, and long-term toxicity effect when inhaled.[55] The functional activity, stability and chemistry of ENPBCs may influence the chemical and toxicity both *in vitro* and *in vivo*. The bio-dissolution of ENPBC which is a measure of bio-durability of chemical and physical properties is a dependent function of size, surface area-shape as well as the medium and ionic strength.[48,53,54] The challenges resulting from these properties have hindered the development of standardized protocols for determining the bioavailability, absorption, distribution, bioaccumulation, metabolism, excretion, release of ENBPCs.

The role of international agencies in engineered nanoparticle (ENP) bio-conjugates (BC)
Toxicity determination and characterization for drug delivery
Accurate and reliable information about the characteristics, concentration, and cytotoxicity of ENPBCs are major properties that can be used to understand the safe use of ENPBC as well as their profile in drug delivery agents. Engineered NPs (ENPs) either naked or coated with bio-conjugates are governed and influenced by their physicochemical properties.[1,48,52-55] The physicochemical

property profiles of any drug delivery are the function of morphological-shape, size distribution, zeta potential, subcellular localization, expression of cell markers, and chemical composition concerning cells' activities. The toxicity of ENPBC, therefore, depends mostly on the physicochemical properties.[1,55] The physicochemical characterization of ENPBCs must be precise and fully determined before assessing their cytotoxicity. The development of regulatory standards and methods for ENPBCs in biological/biomedical applications and systems are urgent mandatory requirements. Various internationals, regionals, cross border organizations, nationals and local organizations have been constituted to provide regulatory guidelines, standards, and safety information for the safe use and discharge of ENPBCs.

The International Organization for Standardization (ISO) main body including the Organization for Economic Cooperation and Development (OECD), Food and Drug Administration (FDA or USFDA), Scientific Committee on Emerging and Newly Identified Health Risks (SCENHIHR) and other regulatory bodies functions is to develop technical safe regulatory guidelines regarding the physicochemical properties of engineered NPs and toxicological assessment[56] which can be used to shape safe usage of nanomaterials before subjecting them to human consumption and other usages. The objectives aimed at describing the inherent nano-toxicity of ENPs and nanomaterials that assure consumer security, safety, and their usage. Furthermore, the ENPs characteristics must be described to detail in terms of their physiochemical properties, impurities, and toxicological levels at *in vitro* and *in vivo*, cellular and molecular level, routes of exposure, uptake, and absorption rates.[17] Other areas of concern are the ethical, legal, and societal implications of ENPBCs and future areas of research.[57] Likewise, the generation of reliable data that supports further development, risk estimation, and potential risks of nanoproducts. However, according to a report by OECD, there are no specific detailed data, regulations available, and standard test methods for ENPBCs human exposure measurement as well as methodologies for risk assessments. Currently, several ISO committee groups are working on various aspects of nano-toxicity and risk assessments. The Nanotechnology Industries Association (NIA),[58,59] ISO-Technical Committees on nanotechnologies have been released for determining the potential biological effects of ENPBCs. More information on NIA standard catalog can be found at https://www.iso.org/ics/07.120/x/.

Due to ENPBCs sizes, ion release, and strong potential ability to functionalized to varying groups of molecules and protein, they have been found to initiate and cause diverse biological effects. Furthermore, these ENPBC-based therapeutics can catalyze properties that overcome biological barriers, and initiate intracellular effective drugs delivery in cells such as macrophages,[3] and targeted disease cells.[3,6] To be sure that ENPBCs are safe,

regulatory and legislative bodies including testing and risk assessment and management bodies must be constituted to guide their usage

Challenges of engineered NPs bio-conjugates
As earlier mentioned, ENPBCs have great potential for improving biomedical research and drug delivery applications.[4] Their successes rely on the ability of surface functionality and stabilization of NPs as drug carriers and as targets to specific cells. However, most challenges facing ENPBC are size specificity and reproducibility. There is no specific equipment responsible for reproducing the exact sizes of ENPBCs and bio-conjugates attachments. The inability to produce exact specific attachments and sizes of ENPBCs have prompted regulatory bodies to examine ENPBCs on a case-by-case-base-effect. This is because different ENPBC nano-sizes have different properties that tend to affect the physicochemical properties and the functionality including pharmacological, immunological, and toxicity profiles. According to a study by Desai,[6] the complexity and nature of ENPBCs may substantially vary and thus, may be difficult to control and predict their behavior in biological systems. It is also difficult to sterilize ENPBC for biomedical applications.[60]

It must also be noted that newer toxicity screening techniques such as xCELLigence always come with their limitations.[61-64] According to Kho et al high capital and consumables costs play a significant role. Secondly, xCELLigence technology work on cells that must adhere to the bottom of microplates and generate viable impedance signals. However, floating/ non-adherent and poorly plated cells may fail to generate signals resulting in false toxicity,[63,64] a common phenomenon of neuronal cells[61,62] and cancer cells due to lack of adherence to the gold microelectrodes. This is likely the main limitation of xCELLigence toxicity testing technology.

On the other hand, the measurement of broth suspended cells and their interactions with ENPBCs using xCELLigence technology is one of the ongoing future technological developments.[64-65] Because non-adherence cells including hematopoietic stem cells by nature have suspension and circulating characteristics[66,67] that have limited their attachment potentials and ability to attach and grow on E-plate wells.[68] However, the attachment characteristics can be enhanced by functionalizing or binding/coating the blood cell-matrix surfaces or the E-plate wells, for example, with peptide immobilization substrates such as fibronectin, laminin, and collagen types I and IV[66-68] that allow for cell adhesion.[66,67,69] Even though xCELLigence operations are designed to work with adherent cells,[8] however, by using binding protein matrices non-adherent cells including those of cancers can now be monitored on xCELLigence technology.[66-68,70] In such instances and/or procedures, the protein matrix binds to cells and enhances their attachment capability on cell well plates. For example, Martinez-Serra et al

improved the attachment capacity of non-adherent cells of hematological malignancies such as leukemia/lymphoma cells with fibronectin. The results showed that the non-adherence leukemia/lymphoma cells were able to attach and grow profusely on the surface of the E-plates wells. In other studies, Abbasalipour et al[71] while testing for transduction characteristics of Lentivirus on K562 cells reported an enhanced adhesion, and multiplication of K562 cells coated with fetal bovine serum. Similarly, Hillger et al[72] while investigating the cellular properties and GPCR drug responses in lymphoblastoid cell lines coated the detector surface with fibronectin to enhance cell surface adhesion that allows for the detection of cellular responses with xCELLigence system. It must be noted that, from several studies, cell adhesion, invasion, proliferation and migration, enhances and changes the local ionic interface at the E-plate gold electrodes such that better cell index impedance is generated with real-time analytical graphical plots. Without this phenomenon of cells attachment and growth in the well plates, the cell index is close to the zero-mark[69] since only viable cells with adherence characteristics potential can benefit from the xCELLigence toxicity enumeration.

Conclusion

Several biomedical benefits accorded to ENPBCs have been applauded by several scholars. As a result, this has advanced research and development in the field of nanotechnology. However, toxicological standards are almost non-existent. The results showed that ENPBCs have different bio-impacts either at the *in-vitro* or *in vivo* animal model levels. These variations are due to differences in the ENPBCs, size compositions, adjustment, storage, route of administration, dose concentration, types of cell lines, target organs, molecular and the model animal used. It is important to generate large data on specific sizes of ENPBC health outcome effects in different settings to estimate and validate the generalizability of specific ENPBC toxicity impact. To keep pace with ENPBCs biomedical products and applications, *in vitro*, *in vivo* assays, clinical trials and long-term impacts are needed to validate their usability and uptake. In addition, more real-time ENPBCs-cell impact analysis using xCELLigence technology is needed to provide significant data for further *in vivo* testing and

subsequent steps in the development of safety standards.

Ethical statement
No ethical approval was obtained because the data needed no human subject interaction. The data were freely available online.

Competing interests
The authors declare no conflict of interest.

Authors' contribution
CSY developed the concept, designed the study, extracted, and reviewed the data, and wrote the initial draft of the article. GSS extracted the data, reviewed the data, and the article. All authors have consented and aligned the article according to Bioimpacts submission guidelines.

Supplementary Materials
Supplementary file 1 contains Tables S1-S2.

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Review Highlights

What is the current knowledge?
√ Traditionally bionanomaterials cytotoxicity exhibits complex toxicity outcomes difficult to interpret.

What is new here?
√ The non-invasive-xCELLigence cellular-bio-response require pooled data to validate bionanomaterials cytotoxicity protocols and methodological development.

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