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Data Mining as a Guide for the Construction of Cross-Linked Nanoparticles with Low Immunotoxicity via Control of Polymer Chemistry and Supramolecular Assembly

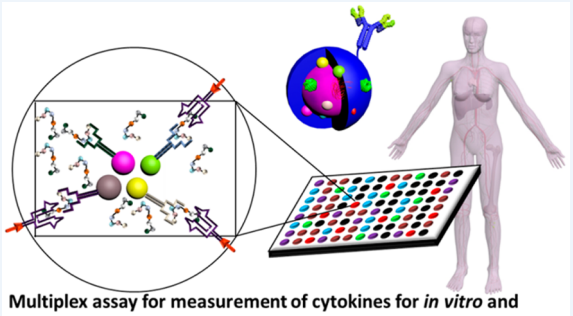
Mahmoud Elsabahy*[,†,‡,§] and Karen L. Wooley*[,§]

[†]Department of Pharmaceutics, Faculty of Pharmacy, Assiut International Center of Nanomedicine, Al-Rajhy Liver Hospital, Assiut University, 71515 Assiut, Egypt

[‡]Misr University for Science and Technology, Sixth of October City, Egypt

[§]Department of Chemistry, Department of Chemical Engineering, Department of Materials Science & Engineering, and Laboratory for Synthetic-Biologic Interactions, Texas A&M University, P.O. Box 30012, 3255 TAMU, College Station, Texas 77842-3012, United States

CONSPECTUS: The potential immunotoxicity of nanoparticles that are currently being approved, in different phases of clinical trials, or undergoing rigorous in vitro and in vivo characterizations in several laboratories has recently raised special attention. Products with no apparent in vitro or in vivo toxicity may still trigger various components of the immune system unintentionally and lead to serious adverse reactions. Cytokines are one of the useful biomarkers for predicting the effect of biotherapeutics on modulation of the immune system and for screening the immunotoxicity of nanoparticles both in vitro and in vivo, and they were recently found to partially predict the in vivo pharmacokinetics and biodistribution of nanomaterials. Control of polymer chemistry and supramolecular assembly provides a great opportunity for the construction of biocompatible nanoparticles for biomedical clinical applications. However, the sources of data collected regarding immunotoxicities of nanomaterials are diverse, and experiments are usually conducted using different assays under specific conditions. As a result, making direct comparisons nearly impossible, and thus, tailoring the properties of nanomaterials on the basis of the available data is challenging. In this Account, the effects of chemical structure, cross-linking, degradability, morphology, concentration, and surface chemistry on the immunotoxicity of an expansive array of polymeric nanomaterials will be highlighted, with a focus on assays conducted using the same in vitro and in vivo models and experimental conditions. Furthermore, numerical descriptive values have been utilized uniquely to stand for induction of cytokines by nanoparticles. This treatment of available data provides a simple way to compare the immunotoxicities of various nanomaterials, and the values were found to correlate well with published data. On the basis of the polymeric systems investigated in this study, valuable information has been collected that will aid in the future design of nanomaterials for biomedical applications, including the following: (a) the immunotoxicity of nanomaterials is concentration- and dose-dependent; (b) the synthesis of degradable nanoparticles is essential to decrease toxicity; (c) cross-linking minimizes the release of free polymeric chains and maintains high stability of the nanoparticles, thereby lowering their immunotoxicity; (d) lowering the amine density for cationic polymers that are being utilized for delivery of nucleic acids lowers the toxicity of the nanoparticles; (e) among neutral, zwitterionic, anionic, and cationic nanomaterials, neutral and cationic nanoparticles usually have the lowest and highest immunotoxicities, respectively; and (f) morphology, dimension, and surface chemistry have a great influence on the ability of nanomaterials to interact with the various components of the biological system and to modulate the immune system.



Multiplex assay for measurement of cytokines for *in vitro* and *in vivo* evaluation of nanoparticle immunotoxicity

1. IMMUNOTOXICITY OF NANOMATERIALS

The potential immunotoxicity of nanoparticles has recently raised special attention because of their ability, like other exogenous materials, to unintentionally interact with various components of the immune system, which is usually harmful (Figure 1).[1−3] Nanomaterials with no apparent in vitro or in vivo toxicity may still trigger various components of the immune system via several mechanisms and mediators. For instance, the development of poly(ethylene glycol) (PEG)-specific antibodies upon administration of PEG-coated liposomes in vivo has recently been observed, which raised questions regarding the approved PEGylated therapeutics that are already on the market for use in humans.[4] Immunogenicity of nanomaterials not only leads to serious adverse reactions that can terminate the therapy but also reduces the therapeutic efficiency.[5] The extent of the

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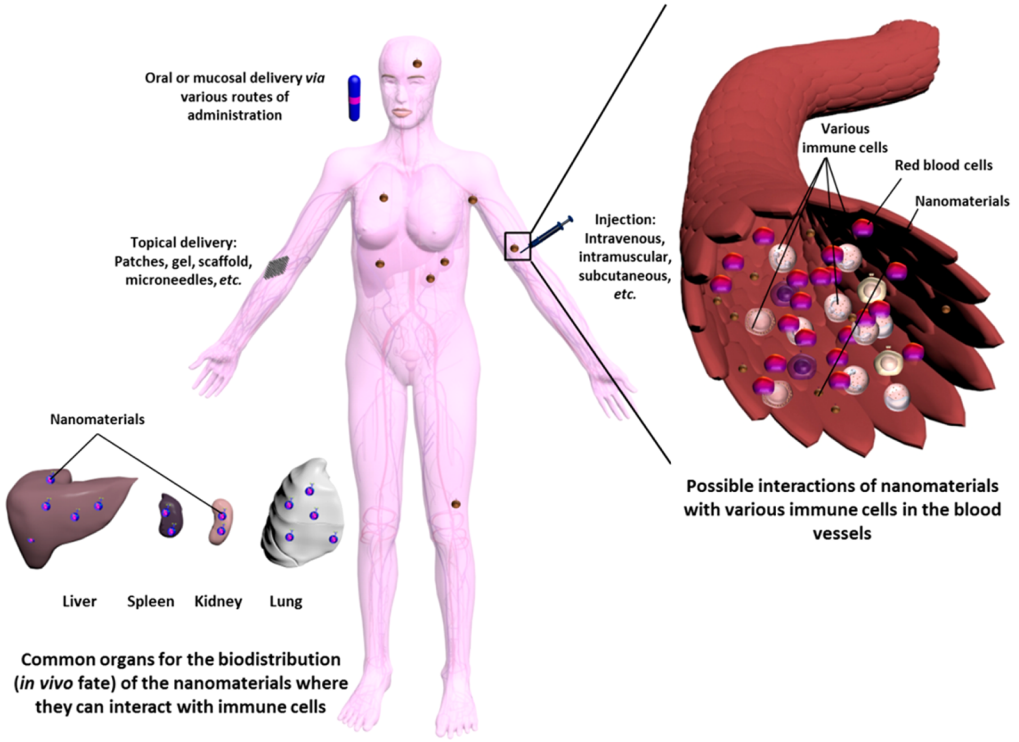

Figure 1. Possible interactions of nanoparticles with the components of the immune system after they enter the body via various routes of administration (oral, mucosal, systemic, or topical). Reproduced with permission from ref 3. Copyright 2013 The Royal Society of Chemistry.

interaction between nanoparticles and the immune system depends on their physicochemical properties (size, morphology, degradability, charge, hydrophobicity, presence of surface-decorating moieties, etc.), their drug payload, and the route of administration.[3]

Although there are several markers (e.g., antibodies, complementary proteins, etc.) for monitoring the modulation of the immune system upon treatment with nanoparticles, proinflammatory cytokines were utilized in this study as biomarkers to compare the immunotoxic effects of nanoparticles of various types. Cytokines are proteins that perform pleiotropic functions and play a pivotal role in regulating the immune system and mediating adverse reactions (e.g., fever, hypotension, nausea), and sometimes deregulation of their expression can be life-threatening.[6,7] Proinflammatory cytokines serve as mediators of inflammatory and immunologic reactions and activate functions of several inflammatory cells during acute inflammatory responses. Hence, it is advisible to monitor the levels of various cytokines to control undesirable responses to nanomaterials. Recently our group has indicated that the induction of cytokines upon treatment with nanoparticles in vitro can be used as a tool to partially predict the in vivo pharmacokinetics of these materials,[8] and others also have found that in vitro results work as a predictor of the in vivo biocompatibility of nanoparticles.[9]

Interactions of nanoparticles of different types with the immune system and plasma proteins have been studied elsewhere.[10−12] However, comparing data from various laboratories and selecting safe and appropriate nanomaterials for a particular clinical application is challenging for several reasons. One of the main reasons is the variation in the experimental setup and conditions, as a result of which the level of expression of a particular marker in the control group itself may have different values in the same type of experiment in different laboratories. This variation becomes evident in immunotoxicity assays, where the expression of up to 100 different biomarkers is measured for nanostructures of various compositions.

Previously, like other groups, we compared either the number of induced cytokines or the levels of induction of cytokines among the different types of nanoparticles and the control untreated group. However, comparing the immunotoxic effects of nanoparticles on the basis of a comparison of the expressions of tens of cytokines is tedious, in particular when one set of nanoparticles induces expression of higher amounts of specific cytokines and lower levels of other cytokines compared with another set of nanoparticles. Hence, in this Account, we have used calculated numerical values that represent the secretion of cytokines using a single value for each set of nanoparticles. These values were found to corroborate the previously published data across types of polymeric systems. Several examples for various types of polymers and nanoparticles are highlighted herein. Only data reported for nanoparticles that did not contain detectable amounts of endotoxins are included. Overexpression of cytokines was calculated only for cytokines with significant differences from the control ($p < 0.05$), and the results are normalized to the value of the control in each experiment. The controls are either untreated cells for in vitro assays or animals injected with saline for in vivo assays. Then the values exceeding 1 (i.e., higher than the control) are summed and used as numerical theoretical values for evaluating the immunotoxicity of the nanoparticles (i.e., the higher the immunotoxicity index value, the higher the predicted immunotoxicity). It is worth mentioning that the concentration of nanoparticles at which the immunotoxicity index is calculated must be provided because the index value depends on the concentration of the tested materials.

The expression of each cytokine and a detailed discussion and explanation of the data are beyond the scope of this

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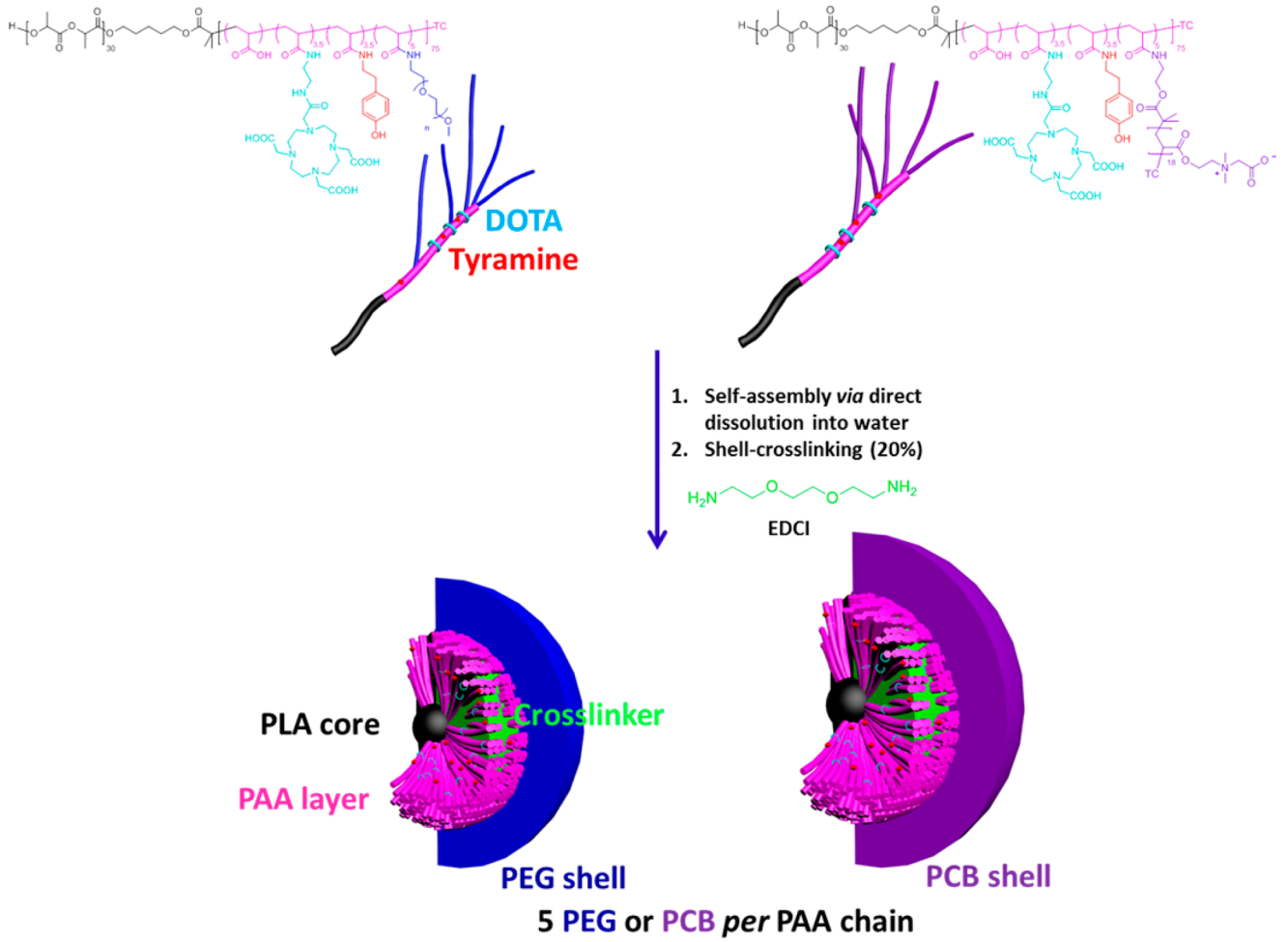

Figure 2. Chemical structures of DOTA- and tyramine-functionalized PEG- and PCB-*g*-PAA-*b*-PLA copolymers and their self-assembly in water and cross-linking to form SCKs with PLA degradable cores, PAA cross-linked shells, DOTA and tyramine as available functionalities, and a hydrophilic shell of either PEG or PCB. TC denotes the $SC(=S)SC_{12}H_{25}$ trithiocarbonate chain end from the RAFT polymerization chemistry. Reproduced with permission from ref 8. Copyright 2013 Elsevier B.V.

Account. Instead this Account highlights the effects of chemical structure, cross-linking, degradability, morphology, concentration, and surface chemistry on the immunotoxicity of an expansive array of degradable and nondegradable polymeric nanomaterials, with a focus on assays carried out using the same in vitro or in vivo models and experimental conditions. It is expected that the data reported in this Account can be utilized as a valuable guide toward the rational design of clinically viable and biocompatible nanopharmaceuticals via control of the synthetic polymer chemistry and supramolecular assembly of various polymeric precursors.

2. EFFECT OF POLYMERIC COATING

We previously synthesized poly(acrylic acid)-*block*-polylactide (PAA-*b*-PLA) copolymers functionalized with 1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA) and tyramine (for potential radiolabeling), and the PAA shell was grafted with PEG (PEG_{5k} or PEG_{2k}) or poly(carboxybetaine) (PCB_{5k} or PCB_{2k}) of various molecular weights (as indicated by the subscripts 5k and 2k, which represent polymer molecular weights of 5000 and 2000 g/mol, respectively) to examine the effect of the polymeric coating and the type of polymer (PEG vs PCB) on the immunotoxicity, pharmacokinetics, and biodistribution of the formed shell cross-linked knedel-like nanoparticles (SCKs) (Figure 2).[8,13] Polymers or nanoparticles were incubated with RAW 264.7 mouse macrophages at a noncytotoxic concentration (500 μg/mL) for 24 h, after which the levels of 23 cytokines (interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (P40), IL-12 (P70), IL-13, IL-17, eotaxin, granulocyte-colony-stimulating factor (G-CSF), granulocyte macrophage-colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), keratinocyte-derived chemokine (KC), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated upon activation normal T-cell expressed and presumably secreted (RANTES), and tumor necrosis factor-α (TNF-α)) were measured utilizing a multiplex assay.[8]

The uncoated nanoparticles resulted in high cellular release of most of the measured cytokines. Functionalization with DOTA and tyramine was also observed to enhance the release of the tested cytokines compared with the unfunctionalized SCKs. PCB_{5k} SCKs significantly induced the production of several cytokines compared with PEG_{5k} SCKs and the control untreated cells, probably as a result of the better shielding efficiency provided by PEG versus PCB polymers to the PAA-*b*-PLA SCKs. Low immunotoxicity was observed for Cremophor-EL, a well-known low-molecular-weight surfactant that is used in several commercial preparations, although it is known to induce

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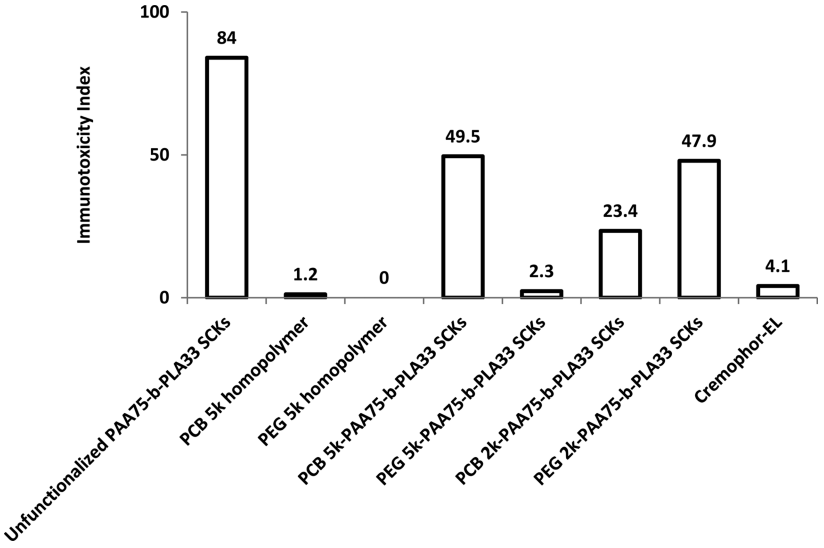

Figure 3. Induction of mouse cytokines IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (P40), IL-12 (P70), IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α following the treatment of RAW 264.7 cells with various polymeric materials. The cytokine induction is expressed by the immunotoxicity index for the polymeric materials tested at a concentration of 500 μg/mL after incubation with the cells for 24 h.

hypersensitivity reactions and peripheral neuropathy in vivo.[14] No cytokine secretions were induced by the free polymers (PEG or PCB) at various tested concentrations. As an extension of these results, it is clear that each nanoparticle should be evaluated per se, as controls of homopolymers may not be comparable in the free and assembled states. It is also evident that steric stabilization is critical in minimizing the toxicity of nanoparticles.

Nanoparticles that had lower in vitro immunotoxicities were correlated with longer blood circulation times and lower clearance in the immune organs (e.g., PEG_{5k} SCKs vs PCB_{5k} SCKs; the detailed information is available in ref 13). The PCB_{2k} SCKs had a similar or slightly better biodistribution profile than the PEG_{2k} analogue, which was also correlated with the higher immunotoxicity of PEG_{2k} SCKs compared with the PCB_{2k} analogues. Higher adsorption of proteins on PCB polymers and nanoparticles compared with PEGylated nanoparticles was also observed. Comparing the types of grafted polymer coating for the PEG_{5k} and PCB_{5k} SCKs is reasonable because of their similar physicochemical characteristics (size and ζ potential values). However, SCKs coated with the 2 kDa PCB had 3 times the size of PEG_{2k}-coated nanoparticles. Hence, the differences in immunotoxicity and characteristics observed for this particular type of nanoparticle (i.e., coated with the 2 kDa PCB chains) might be attributed to the size differences of the nanoparticles rather than the type of polymeric coating. It was also observed that SCKs coated with 2 kDa PEG resulted in massive release of most of the tested cytokines compared with PEG_{5k} SCKs, corroborating the better pharmacokinetic profiles of the nanoparticles coated with PEG of higher molecular weights (the detailed information is available in ref 13) and the better shielding provided by those polymers, as indicated by the almost neutral ζ potential values, compared with the negatively charged surface of nanoparticles coated with the shorter PEG polymeric chains and the uncoated nanoparticles.

In vivo, the PCB-coated nanoparticles resulted in secretions of higher amounts of cytokines compared with the PEG-coated nanoparticles, whereas Cremophor-EL surfactant exhibited slightly higher immunotoxicity compared with PCB-based nanoparticles after injection in mice at a dose of 4 mg/kg (3 h postinjection). Hence, cytokines might be useful as biomarkers for nanoparticle immunotoxicity as well as for partial prediction of in vivo pharmacokinetics and biodistribution profiles.

As a summary of the analyses of the effects of nanoparticle surface chemistry, the high immunotoxicity of charged nanoparticles could be reduced but not eliminated by grafting with hydrophilic neutral or zwitterionic polymers that, as homopolymers, have negligible immunotoxicity. Modification with other functionalities, such as DOTA and tyramine, without polymer grafts further aggravated the immune response to the particles. For this particular type of nanoparticle, PEG-coated nanoparticles appeared to be promising as a drug delivery vehicle with low immunotoxicity both in vitro and in vivo and with a better pharmacokinetic profile in comparison with the PCB-coated analogues. In vitro release of cytokines worked as a partial predictor of the in vivo pharmacokinetics of nanoparticles and was dependent on the type of shell coating, the shielding efficiency provided to the core, and the size of the nanoparticles. In general, the immunotoxicity index is usually lower for in vivo data than for data obtained from in vitro cell-based assays, which might be explained by the different environments and the dilution of the nanoparticles upon intravenous injection. It is worth mentioning that Figures 3 and 4 summarize ca. 60 figures and ca. 190 values (when the data are presented in individual figures for each cytokine) that could be replaced with 11 descriptive values in the two figures (when the data are presented in terms of immunotoxicity indices). In addition, these values are in agreement with the conclusions from the corresponding published data[8] without any need for repetition of the experiments but rather through the application of a simple formula for calculating the immunotoxicity indices of the various tested materials.

3. EFFECT OF SURFACE CHARGE, CROSS-LINKING, AND BIODEGRADABILITY

Although the surface chemistry, degree of cross-linking, and rate of degradation of nanomaterials can be varied according to

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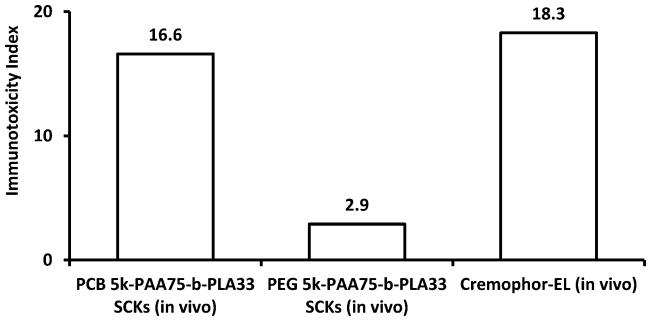

Figure 4. Expression of the 23 mouse cytokines in serum of mice treated with PEG_{5k}- or PCB_{5k}-based SCKs and Cremophor-EL at 4 mg/kg as expressed by the immunotoxicity index (3 h postinjection).

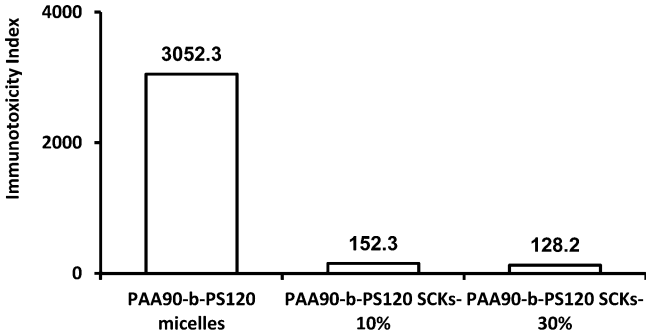

Figure 6. Induction of the 23 mouse cytokines following the treatment of RAW 264.7 cells with PAA-*b*-PS micelles and SCKs with varying degrees of cross-linking. The cytokine induction is expressed by the immunotoxicity index for the polymeric materials tested at a concentration of 32 μg/mL after incubation with the cells for 24 h.

the chemical compositions and structures and there have been attempts to tune those characteristics to meet the needs of cargoes to be delivered and therapeutic applications, it remains an unmet challenge to accurately study the nanomaterial properties as a function of time, especially in complex biological media.[15−17] Nonetheless, it is important to study the effects of these structural modifications, at least taking into consideration the initial starting point (which can be accurately characterized) on the toxicity of nanomaterials. According to recent literature data and as discussed in this section, it is clear that nanoparticles having the collective properties of cationic surface, non-cross-linked unimers, and nondegradable polymers/cross-linkers potentiate stronger interactions with the biological components and induce higher cyto- and immunotoxicities.

In one attempt to synthesize biodegradable nanoparticles with control over their surface chemistry (nonionic, anionic, cationic, and zwitterionic) and with the possibility of cross-linking, polyphosphoester (PPE)-based micelles and cross-linked nanoparticles were prepared using a quick and efficient synthetic strategy.[18] The immunotoxicities of the PPE micelles, SCKs, and their degradation products were studied at a concentration of 5 μg/mL using the same method as described in the previous section.[17] PPE nanoparticles with various surface charges and extents of cross-linking and their degradation products demonstrated high compatibility with RAW 264.7 macrophages, as indicated by low levels of the secreted proinflammatory cytokines, compared with lipofectamine, a commercially available transfection agent that is commonly utilized for delivery of nucleic acids.

When evaluated in terms of the number of induced cytokines, the degradation products of the micelles, the SCKs, and the poly(2-ethylbutylphospholane)-*block*-poly(butynylphospholane) (PEBP-*b*-PBYP) precursor induced minimal immunotoxicities. For the intact micelles, the immunotoxicities were ranked as cationic (13 cytokines) > zwitterionic (7 cytokines) > neutral (3 cytokines) > anionic (1 cytokine), while for the SCKs the immunotoxicities were ranked as cationic (12 cytokines) > anionic (3 cytokines) > zwitterionic (no cytokine induction) (because of the synthetic chemistry approach employed, there was no neutral SCK). Comparing the immunotoxicities of nanoparticles on the basis of the immunotoxicity index rather than the number of induced cytokines demonstrated the same pattern of ranking with a single numerical value for each of the tested nanoparticles (Figure 5). Furthermore, it could provide predictable values for comparison of the immunotoxicities. For instance, the immunotoxicity index of cationic micelles (highest immunotoxicity among the tested formulations) was less than half of the value for lipofectamine (21.3 vs 49.5).

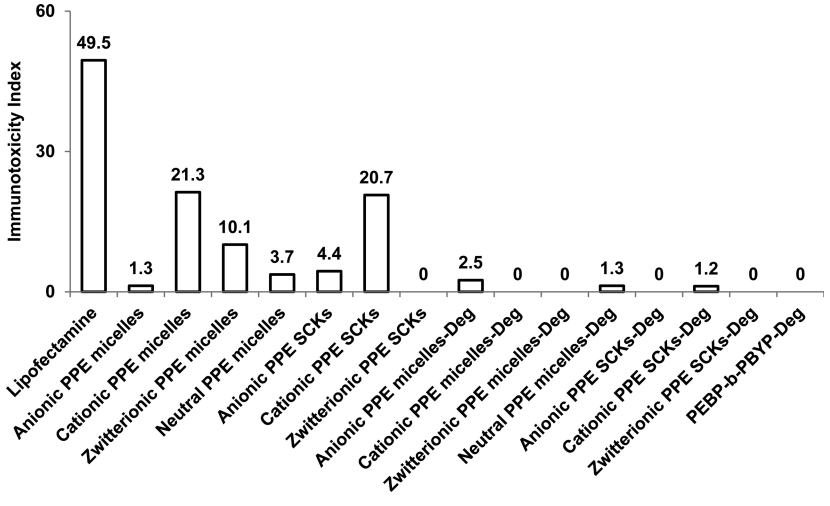

Figure 5. Induction of the 23 mouse cytokines following the treatment of RAW 264.7 cells with PPE micelles and SCKs of various surface charges and their degradation products. The cytokine induction is expressed by the immunotoxicity index for the polymeric materials tested at a concentration of 5 μg/mL after incubation with the cells for 24 h.

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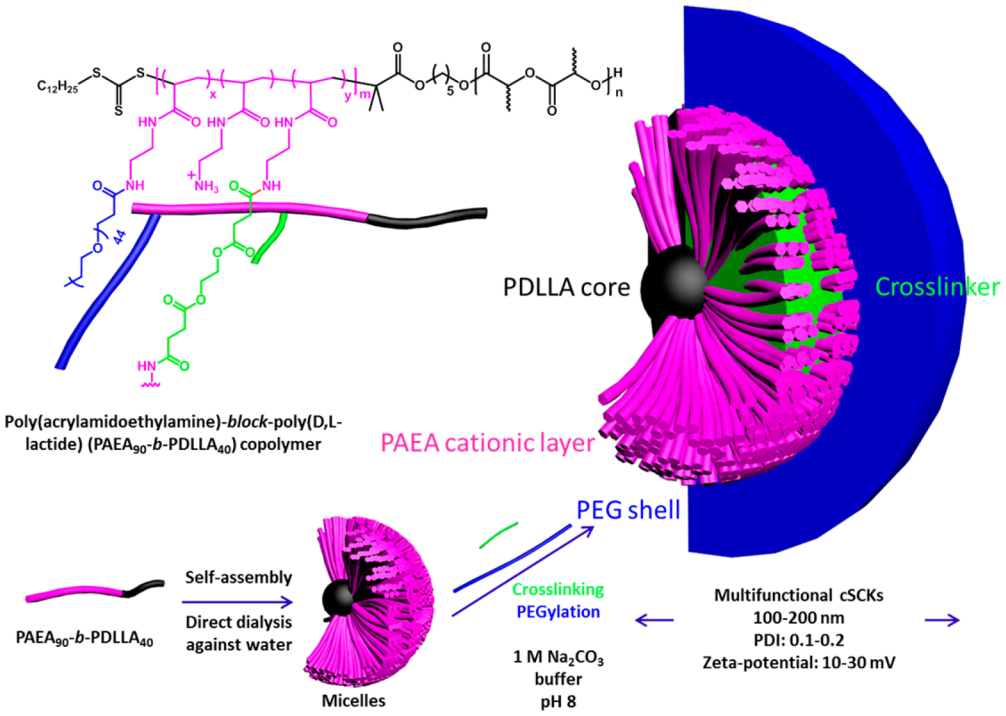

Figure 7. Schematic illustration of the preparation of the micelles and cSCKs via self-assembly of the amphiphilic diblock copolymer $PAEA_{90}$-*b*-$PDLLA_{40}$ followed by cross-linking and/or PEGylation. Also displayed is the overall chemical structure showing the polymer backbone with the possibilities for conjugation with PEG and the cross-linker, where $m = 90$, $n = 40$, and x and y were determined by the particular sample: 5% cSCKs, $x = 0$, $y = 0.05$; PEG micelles, $x = 0.05$, $y = 0$; PEG−5% cSCKs, $x = 0.05$, $y = 0.05$; and PEG−20% cSCKs, $x = 0.05$, $y = 0.2$. Reproduced with permission from ref 24. Copyright 2013 The Royal Society of Chemistry.

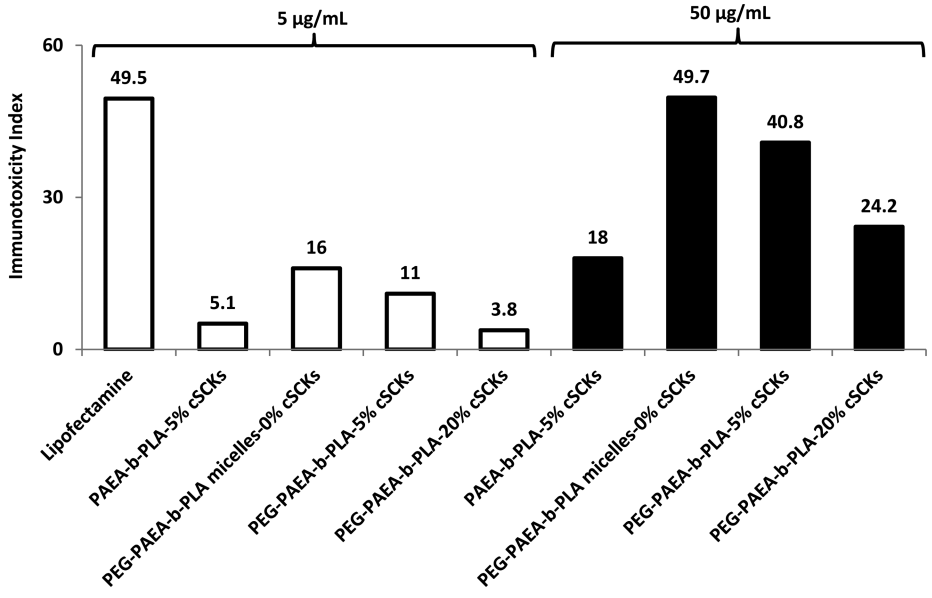

Figure 8. Induction of the 23 mouse cytokines following the treatment of RAW 264.7 cells with lipofectamine, 5% cross-linked non-PEGylated cSCKs, non-cross-linked PEG micelles, and 5% or 20% cross-linked PEG SCKs at 5 μg/mL (white bars) or 50 μg/mL (black bars). The cytokine induction is expressed by the immunotoxicity index for the polymeric materials tested at concentrations of 5 and 50 μg/mL after incubation with the cells for 24 h.

Cationic nanoparticles, even when cross-linked and made up of degradable precursors, induced higher release of cytokines compared with nanoparticles of other surface charges, as is well-reported in the literature, because of their greater capacity to interact with the membranes of cells and with other biological (macro)molecules.[19−21] Cross-linking of zwitterionic micelles to afford the zwitterionic SCKs significantly reduced their immunotoxicities. In vitro degradation data demonstrated the superior stability of the anionic SCKs compared with the anionic micelles. The increased immunotoxicity of anionic micelles upon cross-linking might be due to the higher stability of the cross-linked micelles toward production of the degradation products, which induce minimal toxicity. It is important that the intact nanoparticles and their degradation

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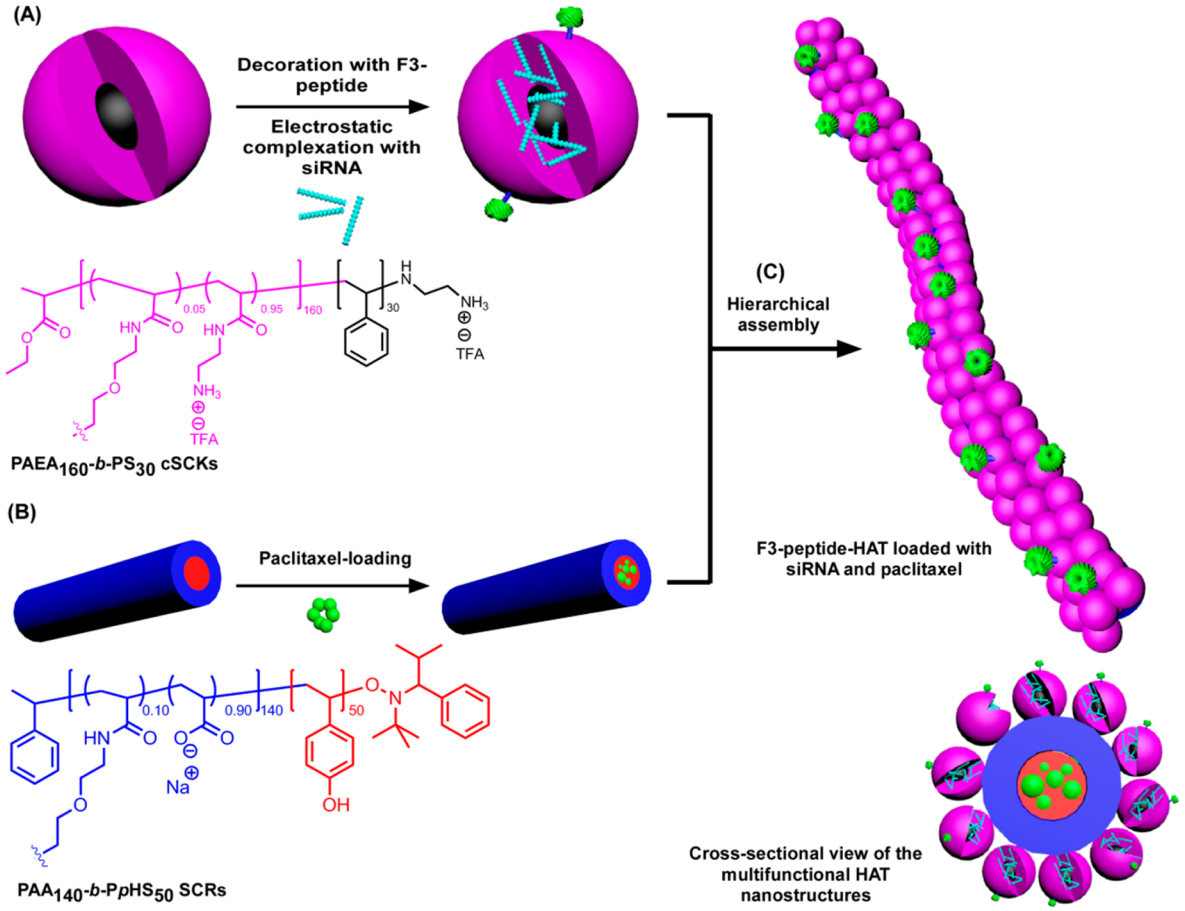

Figure 9. Construction of HAT nanostructures as a template for the codelivery of siRNA and paclitaxel: (A) Electrostatic complexation of $PAEA_{160}$-*b*-PS_{30} cSCKs and nucleic acids (e.g., siRNA) and decoration of the surface of nanoparticles with targeting ligands (e.g., F3 peptides); (B) Loading of hydrophobic drugs (e.g., paclitaxel) into SCRs composed of PAA_{140}-*b*-P*p*HS_{50}. (C) Hierarchical assembly of cSCKs and SCRs to form the HAT nanoassemblies and a cross-sectional view of the multifunctional HAT nanostructures. Reproduced from ref 29. Copyright 2013 American Chemical Society.

products be studied independently, as illustrated in the data summarized in Figure 5 and in other reports. For instance, a degradation product from the cationic degradable PPE-based nanoparticles imparted an anti-inflammatory character serendipitously by efficiently inhibiting the transcription of inducible nitric oxide synthase and preventing the overproduction of nitric oxide, although the nanoparticles were not loaded with any specific therapeutic drug.[22]

Nondegradable poly(acrylic acid)-*block*-polystyrene (PAA-*b*-PS) micelles were prepared and subsequently transformed into SCKs by cross-linking of the shell domains of the micelles via amidation chemistry, with nominal cross-linking of 10% or 30%, for delivery of cisplatin.[23] The effect of cross-linking on the immunotoxicity of the nanoparticles was tested using the same method as described previously. Nanoparticles either free or loaded with cisplatin were not immunotoxic at low concentration (ca. 0.08 μg/mL), whereas high secretion of cytokines was observed for both micelles and SCKs at a concentration of 32 μg/mL (Figure 6). According to the method of analysis used in the study at that time, there was an increase in immunotoxicity with increasing degree of cross-linking. After reanalysis of the data and calculation of the immunotoxicity index, it was found that the immunotoxicity of the micelles decreased with increasing degree of cross-linking.

In another study, comparisons of the effects of PEGylation side-by-side with the degree of cross-linking were investigated. Poly(acrylamidoethylamine)-*block*-poly(D,L-lactide) ($PAEA_{90}$-*b*-$PDLLA_{40}$) copolymers were synthesized and self-assembled in water to yield micelles (Figure 7).[24] The effects of grafting PEG on the micelle surface and the degree of cross-linking of the PAEA layer on the immunotoxicity were studied. Lipofectamine induced significantly higher expression of almost all of the tested cytokines compared with the cationic SCKs (cSCKs), PEGylated or not (Figure 8). The general conclusion after calculation of the immunotoxicity index is that both PEGylation and cross-linking are important to reduce the immunotoxicity of nanoparticles. Although there could be several contributing factors, the reduced immunotoxicity is expected to be mainly due to the limitations of the flexibility of the shell with cross-linking and the hydrophilic layer that surrounds the cationic nanoparticles, both of which hinder the nanoparticles from rapid dissociation and interaction with the surrounding biomolecules. Increasing the concentration of nanoparticles, regardless of the degree of cross-linking, was generally associated with enhanced levels of the secreted cytokines, which is in agreement with dose-dependent immunotoxicity of nanomaterials.[25,26]

4. EFFECT OF STRUCTURAL MODIFICATIONS ALONG THE POLYMER BACKBONE

For the delivery of nucleic acids, cSCKs with a PS core and a PAEA shell were prepared for efficient electrostatic complexation

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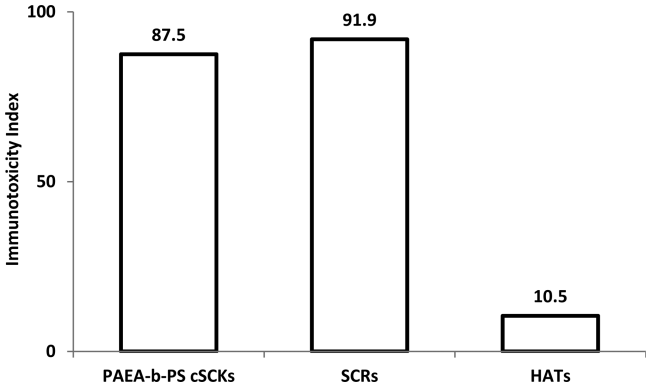

Figure 10. Induction of the 23 mouse cytokines following the treatment of RAW 264.7 cells with cSCKs (5 μg/mL), SCRs (0.5 μg/mL), or HATs (5 μg/mL) for 24 h. The cytokine induction is expressed by the immunotoxicity index for the SCRs (0.5 μg/mL) and cSCKs and HATs (5 μg/mL) after incubation with the cells for 24 h.

with negatively charged nucleic acids.[27] Incorporation of varying amounts of low- and high-pK_a histamine and primary amines into the shell was performed to test the effect on the toxicity and transfection efficiency of the resulting nanoparticles. Histamine was incorporated in order to increase the buffering capacity of the nanoparticles and thus enhance their transfection efficiency. The immunotoxicities of the 0% and 15% histamine cSCKs were studied by measuring the levels of the 23 cytokines upon treatment of RAW 264.7 mouse macrophages with the nanoparticles for 24 h. Generally, lower secretion of the cytokines was observed from cells treated with the histamine-modified cSCKs compared with the cSCKs with 100% primary amines. A similar trend was observed for most of the tested cytokines, which indicates the potential immunotoxicity of the nanoparticles with higher primary amine density (i.e., 0% His cSCKs). The incorporation of histamine into the polymer precursor significantly reduced the cytotoxicity of the nanoparticles, as indicated by the higher IC_{50} value of the 15% His cSCKs compared with that of the 0% His cSCKs (20.7 vs 16.3 μg/mL, $p < 0.05$). The reduced cyto- and immunotoxicity of the histamine-modified cSCKs might be due to their limited ability to interact with the cell membrane and stimulate the secretions of cytokines, which might result from the lower charge density along the cationic polymer backbone.

5. EFFECT OF MORPHOLOGY AND CONTROL OF POLYMERIC NANOSTRUCTURES VIA SUPRAMOLECULAR ASSEMBLY

Few studies have focused on the relationship between the nanoparticle shape and the cytokine release pattern either in vitro or in vivo, which is challenging as it depends on several variables, including the type of cell line and the animal model. For instance, in comparison of spherical and sheetlike zinc oxide nanoparticles with approximately similar specific surface areas, the spherical nanoparticles induced higher and lower release of TNF-α in RAW 264.7 mouse macrophages and mouse primary dendritic cells, respectively, compared with the sheetlike nanoparticles.[28]

Multifunctional hierarchically assembled theranostic (HAT) nanostructures were previously constructed via templating of cationic spherical nanoparticles (cSCKs) onto anionic shell cross-linked rodlike nanoparticles (SCRs) (Figure 9).[29,30] The functionalities of these HAT nanostructures include a cationic

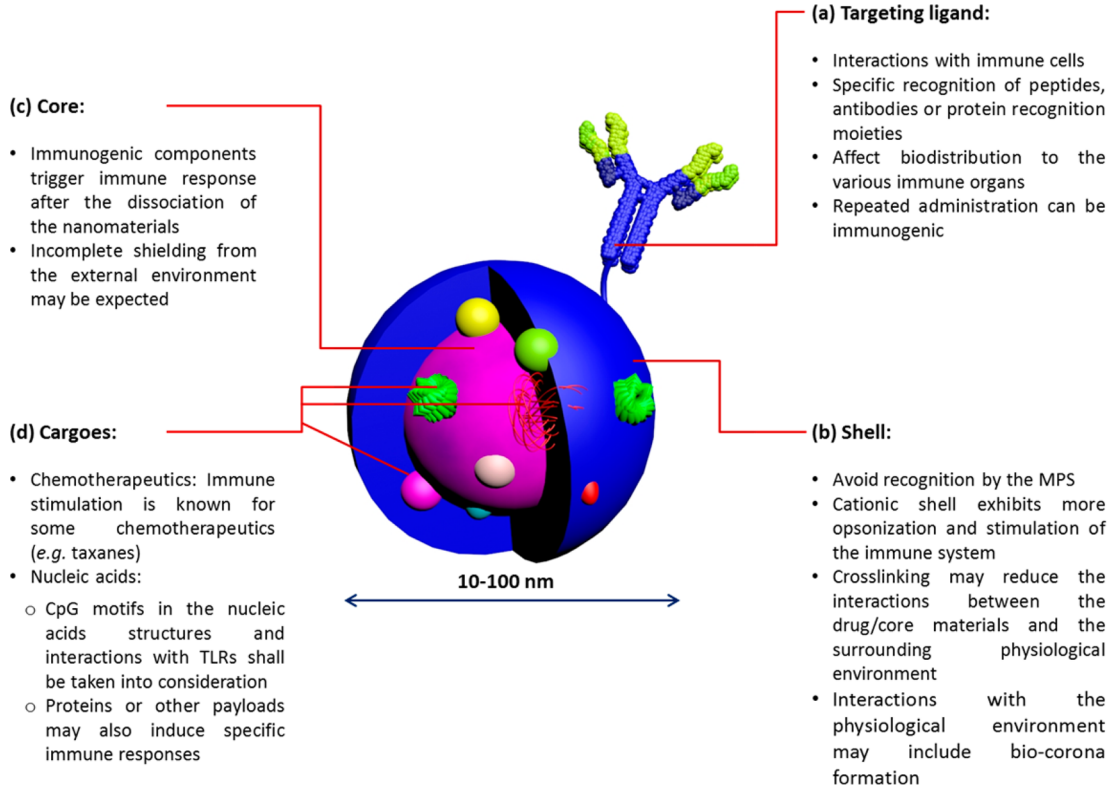

Figure 11. Illustration of the general composition of a multifunctional nanoparticle for biomedical delivery applications, highlighting some important considerations for the design of nanoparticles of low immunogenicity. Reproduced with permission from ref 3. Copyright 2013 The Royal Society of Chemistry.

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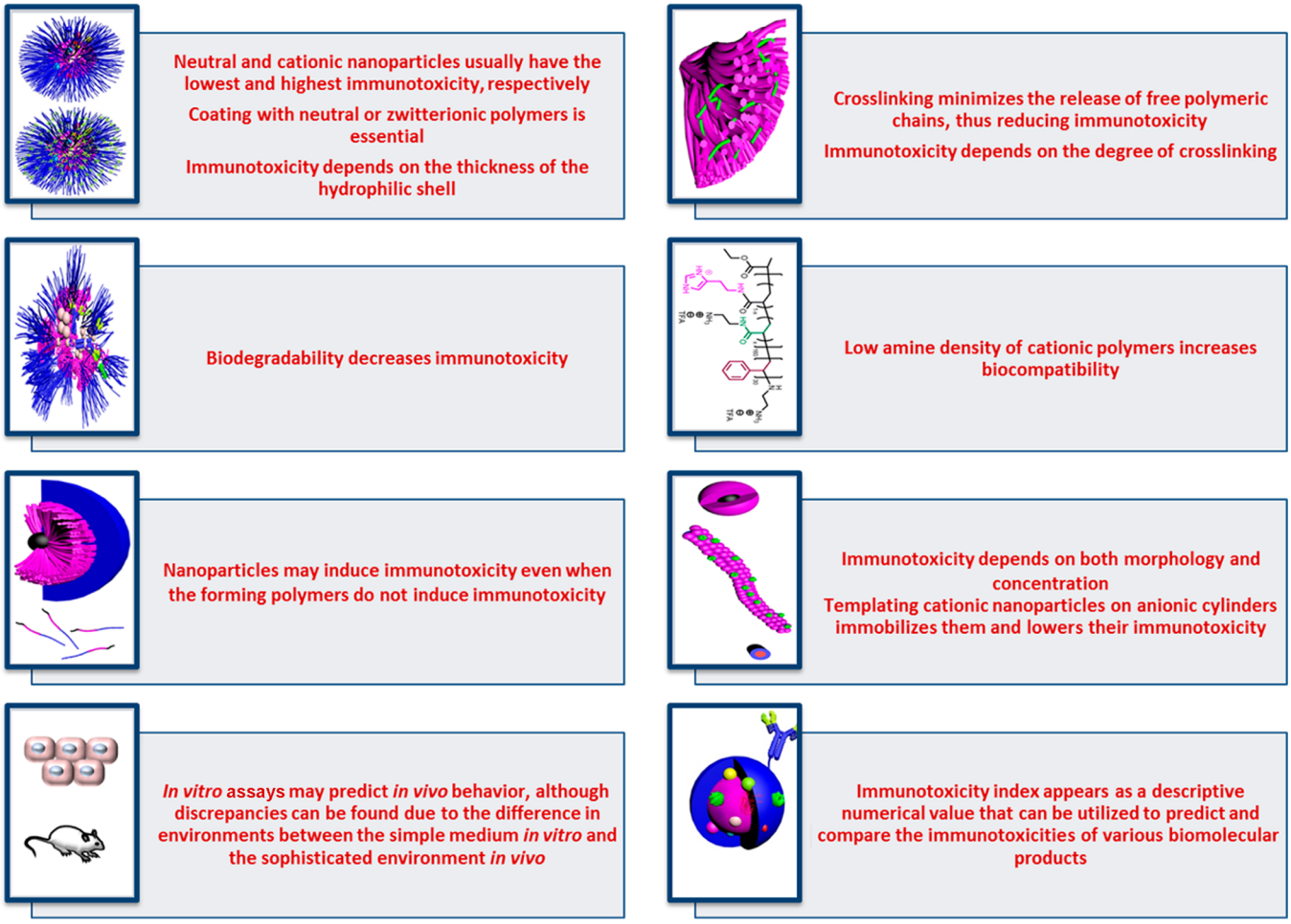

Figure 12. Summary of the important features that should be considered for the future design of clinical nanopharmaceutical products.

surface for complexation of nucleic acids (e.g., small interfering RNA (siRNA)) and surface decoration with active targeting moieties (F3-tumor homing peptide), anionic cylinders for solubilization of hydrophobic anticancer drugs (paclitaxel) in their cores, sites for radiolabeling, and elongated morphologies for potential prolongation of the blood circulation time after in vivo administration. Although beyond the scope of this Account, these sophisticated polymeric structures were found to have a unique "stick and trigger" intracellular trafficking mechanism via binding to cell surfaces with the cationic spheres followed by intracellular release of these spheres while the cylindrical parts remained near the cell surface, which allowed for exceptional transfection efficiency.

The immunotoxicities of cSCKs and HATs (each at 5 μg/mL) and anionic cylinders (0.5 μg/mL) were studied by the same method as described previously. Generally, the highest secretion of cytokines was observed from cells treated with the cylinders, followed by cSCKs, whereas the HATs were found to be relatively the least immunotoxic (Figure 10). The strong induction of cytokine release from cells upon treatment with anionic cylinders (0.5 μg/mL) was surprising and could be explained by similarities to bacterial dimensions and surface properties.[31] An increase in the release of cytokines from cells treated with the cylinders was also observed with an increase in the concentration of the nanoparticles. Spherical cationic nanoparticles usually induce the secretion of high amounts of cytokines as a result of their nonspecific binding to cell membranes and also probably their similarities to viral dimensions.[31] Binding of these cationic spheres onto the surface of the anionic cylinders (i.e., HAT nanostructures) reduced the release of the cytokines, perhaps for two main reasons. First, lowering the charge density and limiting the free movement of the cationic nanoparticles should afford less possibility for interactions between nanoparticles and the cell membranes and biomolecules in the medium. Second, masking the bacterial-like properties of the negatively charged anionic cylinders should reduce the immunostimulatory effects of the unbound anionic cylinders. The lower immunotoxicity of HATs was associated with higher efficiency in siRNA binding and transfection efficiency compared with the cSCKs[29,30] in addition to their potential ability to carry more than one cargo and extend the blood circulation time in vivo.[32] The calculation of the immunotoxicity index was in accordance with the conclusions of this study.

6. CONCLUDING REMARKS

The immunotoxicity of nanomaterials must be evaluated on an individual basis, as it depends on the overall composition of the nanoparticles in addition to the embedded cargoes and moieties utilized to decorate the surface of nanoparticles (Figures 11 and 12). However, on the basis of the collected data, it is advisible to construct nanoparticles from degradable precursors and to impart nanoparticles with greater kinetic stability via cross-linking of some of their components to a degree that must be optimized for each type of nanoparticle.

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Shielding the cores of nanomaterials with a neutral layer of PEG continues to be the most efficient way to prepare stealth nanoparticles with minimal immunotoxicity, although the safety of PEGylated biotherapeutics remains controversial. The length of the polymer grafts that constitute the hydrophilic shell and the degree of cross-linking are among the most important parameters to be controlled precisely. Morphology and surface chemistry also appear to contribute significantly to the capacity of nanomaterials to interact with the surrounding biomolecules in the biological environment. Future studies should be designed to take into consideration the effects of overall particle mechanical properties, which may also be a contributing factor to immunotoxicity and other biological responses. Such studies would require that the particle size, shape, and surface chemistry be held constant while only the particle rigidity is altered.

In some cases, in vivo administration of even low doses of nanoparticles may induce hypersensitivity reactions and biochemical changes through "complement activation-related pseudoallergy".[33] There are also several attempts to delay the detection of nanomaterials by the immune system. For instance, Discher and co-workers designed nanobeads decorated with CD47 peptide as a "marker of self" to mimic "self" cells and thus avoid recognition by the immune system.[34] Upon intravenous injection, the attached peptide delayed the clearance and prolonged the blood circulation time of the nanoparticles and allowed for higher accumulation in the tumor tissues of treated mice.

Finally, the immunotoxicity index appears as a descriptive numerical value that can be utilized to partially predict and simply compare the immunotoxicities of various biomolecular products. The most attractive feature is that from one numerical value (the immunotoxicity index), to a great extent, the immunotoxicity levels of of several nanomaterials could be expressed and compared (as long as the measurements were conducted at equivalent concentrations). At the time when these papers were published, the immunotoxicity index was not available, and the explanation of the data was much more complicated. We expect a much easier understanding and corroboration of experimental data in the future, and thus, the information summarized in this Account will provide useful guidelines for the design of clinically viable nanopharmaceutical products of high efficiency and low toxicity and immunogenicity.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: mahmoud.elsabahy@chem.tamu.edu.
*E-mail: wooley@chem.tamu.edu. Tel.: +1 979 845 4077. Fax: +1 979 862 1137.

Notes

The authors declare no competing financial interest.

Biographies

Mahmoud Elsabahy is Director of the Assiut International Center of Nanomedicine at Alrajhy Liver Hospital, Assistant Professor at the Department of Pharmaceutics, Faculty of Pharmacy, Assiut University, Egypt, and Assistant Director of the Laboratory for Synthetic-Biologic Interactions at the Department of Chemistry, Texas A&M University, Texas, USA. He received a B.S. in pharmaceutical sciences from Assiut University, and then he finished his M.Sc. and Ph.D. degrees from the Faculty of Pharmacy, University of Montreal, Canada. His research interests include the rational design and evaluation of pharmaceutical nanocarriers for delivery of therapeutics and diagnostics via various routes of administration, for which he has received several prestigious awards. He is one of the members of the Senior Professors Council at Misr University for Science and Technology, Egypt. He is also one of the first 15 official members of the national "Egyptian Young Academy of Science" (Academy of Scientific Research and Technology, Ministry of Scientific Research, Egypt).

Karen L. Wooley received a B.S. in chemistry from Oregon State University in 1988 and a Ph.D. in polymer/organic chemistry from Cornell University in 1993. She began as an Assistant Professor at Washington University in St. Louis in 1993, was promoted to Full Professor in 1999, and was installed as a James S. McDonnell Distinguished University Professor in Arts & Sciences in 2006. In 2009, she relocated to Texas A&M University, taking a position as the W. T. Doherty-Welch Chair in Chemistry, and was awarded the title of University Distinguished Professor in 2011. Her research areas include the synthesis and characterization of degradable polymers, unique macromolecular architectures, and complex polymer assemblies and the design and development of well-defined nanostructured materials, for which she has received several awards, including the 2014 American Chemical Society Award in Polymer Chemistry, a 2014 Royal Society of Chemistry Centenary Prize, and the 2015 Oesper Award. She serves as an Associate Editor for the *Journal of the American Chemical Society*.

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