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High-resolution ICP-MS approach for characterization of magnetic nanoparticles for biomedical applications

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ABSTRACT

The potential of iron oxide-based nanoparticles (IONs) as theranostic agents is believed to be in a great part due to non-invasive diagnosis and therapeutic applications. However, there is still a lack of well-recognized methodology to assess bioresistance, hypotoxicity, reactivity toward pertinent biomolecules, as well as an eventual dose of IONs as prerequisites for their clinical use. In this study, we demonstrate how application of high-resolution ICP-MS in combination with conventional ultrafiltration can address these important issues in a simple and high-throughput way. Based upon interference-free and sensitive measurements of iron and sulfur isotopes ensured by sector-field ICP-MS mode, the comparative testing of a series of novel IONs modified by PEG or PEG and an ionic liquid, was performed. Satisfactory stability (less than 1 % of soluble Fe), minor toxicity (by virtue of releasing a free iron) and transit into bioconjugates in human serum, different in speed, proved the prospective of the tested IONs for in-depth preclinical development.

1. Introduction

There is a growing demand for advanced nanoscale diagnostic and therapeutic tools and among these, iron oxide-based nanoparticles (IONs) are arguably the most advanced nanomaterials to afford improved diagnostic imaging, drug delivery and therapeutic applications [1–6]. Specifically, the therapeutic efficacy of IONs stems from their ability to target the diseased tissue, activate a drug, locally produce a temperature increase (following, or not, the application of an external source of energy), modify genes, replace diseased cells by stem cells, etc. [7], as well as due to intrinsic tumor-inhibiting action [8]. This makes them potentially useful for treatment of a wide range of different diseases. Furthermore, numerous in vitro and in vivo studies have demonstrated the safety of medicinal use of IONs, particularly, in case of polymer-coated formulations [9,10].

Nonetheless, despite the appeal of IONs for both diagnostic and therapeutic applications, there is relatively little progress toward translation to clinical applications. Indeed, while a great variety of IONs were synthesized and tested in the past decade, including multifunctional materials [8], only a few nanomedicines have been commercialized for treatment of iron-deficient anemia and cancer, e.g., Venofer® and Nanotherm®, respectively, as well as for tumor imaging (Feridex I.V.®, Feraheme®, Endorem®, etc.) [11]. The reasons of the delayed use of IONs for human treatments are multi-fold. First, it is hard to identify a nanosystem that is better than others as each research group tries to prove the advantages of a nanomaterial on which it is working, compared with others. Furthermore, in the academic world, the development of ever new formulations published in high-impact journals is rewarded much greater than pursuing clinical development of existing formulations. Next, the characterization of new IONs for biomedical applications usually covers the topics of the dimensionality, chemical composition of the inorganic core and the organic shell, and the resulting magnetic properties [12] but lacks understanding of the interactions of these materials in biological media [13,14]. Lastly, screening and preclinical development programs in use for IONs (as well as other nanomaterials) are not systematically optimized and for the most part are deficient in assessing pharmacological properties such as stability in physiological medium, bioresistance, nontoxicity, an inclination to biotransformations in human blood serum, etc. Notably, a deficiency in any such quality would disqualify a nanomaterial yet at the stage of preclinical examination.

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It is the pharmacological component often being overlooked that motivated us to develop an analytical approach which could help establish a screening framework for IONs, preferably adaptable in routine bioanalytical laboratories. Sector-field ICP-MS was chosen as a quantification tool for the principal elements, iron and sulfur, as a part of nanomaterial core and pertinent blood biomolecules, respectively, due to method's proven ability to circumvent spectral interferences as well as matrix interferences from complex biological milieus as human serum [15,16]. By taking advantage of its simplicity and adaptability, we applied here common ultrafiltration to separate the nanoparticulated and soluble fractions of iron and sulfur and to obtain the filtrates amenable to direct analysis. The merits of such sample preparation, using 100 and 30 kDa cut-off filters, have been demonstrated recently using a single sort of IONs [17]. With the objective of promoting the developed ultrafiltration-ICP-MS assay as a high-throughput screening platform for medicinal nanoparticles, we prepared a series of novel IONs by an original method using PEG (or PEG and an ionic liquid, IL)–inorganic salt–water systems [17] and compared them in terms of appeal to further preclinical testing.

2. Experimental

2.1. Chemicals and materials

The IONs were synthesized and coated according to a facile one-pot procedure reported previously [18]. In more detail, the modification of this procedure, as well as particle characterization, is described in the Electronic supporting information (ESI). The resultant ION suspensions were diluted with 10 mM phosphate buffer (pH 7.4) containing 100 mM NaCl (PBS), to give 2×10^{-4} M Fe in final standard suspension. Amicon Ultracel units with a cut-off molecular mass of 30 or 100 kDa used for ultrafiltration experiments were purchased from Millipore (Molsheim, France).

All reagents were of analytical grade and products of Sigma-Aldrich (St. Louis, MO, USA), including human serum (from human male AB plasma; total protein concentration 40–90 g/L). High purity water obtained from a NANO Pure purifier system (Thermo Fisher Scientific, Waltham, MA, USA) was used.

2.2. Sample preparation

Each standard suspension of IONs was added to an equal volume of PBS or human serum solution and a mixture, composed of 1×10^{-4} M Fe in PBS or 10-fold diluted serum, was incubated at 37 °C. Aliquots (0.5 mL) were periodically taken over 1 h, placed in an ultracentrifugation unit, and centrifuged for 15 min at 10 000 rpm at 0 °C. The filtrates were diluted (1:10) with 3 % HNO_3 and taken for further analysis.

2.3. Analysis and data acquisition

An Element 2 instrument (Thermo Fisher Scientific) was used for ICP-MS measurements. The total iron and sulfur in acidified samples were determined using the following instrumental settings: plasma gas flow, 14 L min^{-1}; auxiliary gas flow, 0.9 L min^{-1}; nebulizer gas flow, 0.9 L min^{-1}; analyzed sample flow, 0.8 L min^{-1}; RF power, 1200–1300 W; dwell time, 1 ms. External calibration was done against calibration curves prepared using one-element standard solutions (High-Purity Standards, North Charleston, SC, USA) in the range of 0.1–100 μg L^{-1} Fe and 1–1000 μg L^{-1} S, and In as an internal standard to monitor instrumental plasma stability. Working with diluted serum samples ensured no substantial salt built-up in sample introduction system or matrix effect on instrumental sensitivity. Concentrations of soluble (non-particulated) iron species and a total of sulfur-containing serum compounds were calculated as the iron or sulfur concentration in filtrates, respectively, with due account for the blank signals of serum (in case of toxicity testing) and IONs. Although sector-field ICP-MS measurements enable tackling spectral interferences for the monitored iron (^{57}Fe) and sulfur (^{32}S) isotopes and hence bridging the gaps in resolution and sensitivity distinctive to quadrupole-based instruments, the acquired results were validated against the data of ICP-optical emission spectroscopy analysis (the operating parameters are listed in Table S1).

Table 1
Characteristics of IONs prepared using different coatings and ammonium salts.[a]

Synthesis system	C_{Fe} in suspension (mg L^{-1})	Soluble C_{Fe}	
		(μg L^{-1})	(%)[b]
PEG–ammonium sulfate	0.91 ± 0.05	9.6	1.1
PEG–sodium nitrate	0.99 ± 0.08	10.0	1.0
PEG–sodium acetate/carbonate	1.21 ± 0.09	7.0	0.6
PEG–IL–sodium nitrate	1.22 ± 0.08	5.1	0.4

[a] From 3–5 different syntheses.
[b] As a part of total particulated iron.

3. Results and discussion

3.1. Characterization of IONs

The size, shape and charge of the IONs stabilized with PEG and IL have been investigated as described in detail in the ESI using common characterization techniques. Briefly, these measurements showed that most IONs are nearly spherical and roughly monodisperse with a primary particle size in the range of 10–20 nm and approximate zeta potential values ranging from −23 to −27 mV in PBS (see Figs. S1–S5).

Other key questions to be answered when characterizing novel nanoparticles include: What is the concentration of iron in a suspension; and how repeatable is the ION preparation in terms of iron content? The iron concentration in standard suspension is important to knowing the amount of material added to human serum (see below) or animal subjects. In its turn, variations in iron content may originate from every synthetic/washing-out step (as detailed in the ESI) and should be minimized, especially if the scalable production of IONs is the task (the standardization and a large-scale production remain a bottleneck in their clinical development).

Table 1 gives the concentration figures of interest determined in suspensions diluted with PBS and acidified right after the synthesis. In this way, any possible effect of storage or the phosphate medium on particle stability [17] was avoided. From the data of Table 1, it is evident that for each type of IONs the synthesis repeatability (in terms of iron content) is acceptable, as RSDs do not exceed 8 % even for particles prepared at different days. The 'best' case regarding the deviation of actual Fe concentration from the theoretical value (as from iron salts taken for synthesis (see the ESI); 1.0 mg L^{-1}) is presented by the PEGylated IONs prepared in the nitrate medium. It is worthwhile to note that for the IONs, originating from the sulfate medium, the background signal of ^{32}S was too high (0.64 mg L^{-1}) to include the material in further biotransformation studies.

3.2. Particle stability in PBS

Previously, it was reported by Jedlovszky-Hajdú et al. [19] and Kuznetsova et al. [17] that IONs even stabilized with polymers can exhibit instability effects in PBS and the stability problem is related to the nature of the buffer. The most plausible explanation is behind the polymeric structure that does not completely coat the magnetite surface, leaving free Fe—OH groups to be confronted by the phosphate groups. Therefore, we deemed it mandatory to prove that the IONs under investigation are sufficiently stable against

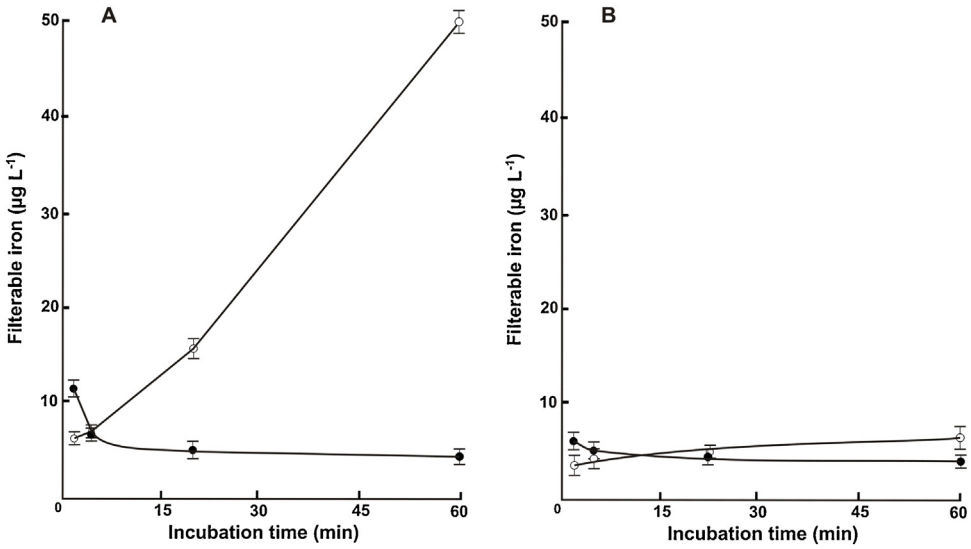

Fig. 1. Degradation of IONs, coated with (A) PEG and (B) PEG and IL, in human serum as a function of time. Black and open circles show the traces for low and high molecular-weight iron, respectively. In the latter case, the iron blank signal for untreated diluted serum (14.7 ± 0.4 μg L^{-1}) is taken into account. Both types of particles were obtained from the nitrate system and analyzed in triplicate.

iron leaking yet before introducing them into a harsher environment of human serum. As can be seen in Table 1, the concentration of Fe in the filtrates obtained after the filtration through 30 kDa cut-off filters is in the low μg-per-liter range, comprising thereby less than 1 % of total particle-core iron which is quite an adequate stability indicator. It should be mentioned that the amount of released ionic metal slightly varies over the recorded period of time (0–30 min; see Table S2) and the values presented in the table correspond to a maximal release due to the phosphate reactivity.

3.3. Bioresistance of IONs

In human serum, aside with becoming enveloped by proteins, IONs would face a complex serum matrix that might further compromise their stability. In experiments designed to verify this effect, we applied IONs at a concentration of 1×10^{-4} M Fe (in 10 times diluted human serum) to imitate an average dose of 1–2 mg kg used in in vivo studies on experimental animals [20,21] (see [17]. for calculations). The resulting samples were analyzed as described in Section 2.2, and Fig. 1 displays time evolution of soluble iron species. To infer these dependences, it is important to comprehend that as the IONs degrade into dissolved iron ions, the latter are sequestered in iron storage proteins [22]. Therefore, in the case of IONs stabilized only by PEG (Fig. 1A), a more significant variation was observed for high molecular-weight iron, as a result of the formation of iron-containing sequestration compounds.

Major differences in stability can be observed as a function of the coating from the comparison of panels A and B in Fig. 1. Evidently, additional shielding of particle surface by the IL led to protection of Fe—OH groups against serum phosphate (and possibly other iron-coordinating ligands) and thereby to a greater compositional constancy. On balance the iron species released from IONs during a short residency in human serum (<20 min) constitute only a fraction of original material (≤1.2 %), being in effect at the same level as in PBS (see Table 1). Physiological importance of such short time is explained by the characteristic timescale of ION circulation in the blood, which comprises only a few minutes (e.g., for commercialized Resovit® and Endorem®, the blood half-lives are 5 and 6 min, respectively [23,24]).

It should be pointed out that the production of ionic iron species within serum can be regarded ambivalently for in vivo applications. On the one hand, there are data showing that iron overload exhibits toxicity in humans only at a concentration above 60 mg Fe per kg [25]. On the other, non-protein bound iron, even when present in trace amounts, can induce oxidative stress due to its ability to catalyze the formation of reactive oxygen species [26]. Anyway, our quantitative measurements revealed the existence of the majority of Fe in the nanoparticulated form (≥99 %), and a fairly slow release of iron converted into protein adducts may be offset by the clearance from circulation by endogenous pathways.

3.4. Binding to serum biomolecules

While a well characterized phenomenon (see e.g. [14] and references therein), the formation of the biomolecular corona, an inevitable event for IONs in human serum, has a mixed character in terms of biomedical implications. When covered with serum proteins. IONs exhibit a lower uptake (compared with the 'naked' particles), which by one account makes them less suitable for targeted drug delivery or visualization. On contrary, IONs bearing a protein corona might better escape the immune system, cross biological barriers and being larger in size, avoid rapid renal clearance and hence possess enhanced circulation.

In our case, the PEGylated IONs lose their synthetic identity very fast and after 10-min exposure to serum are covered by biomolecules (Fig. 2, lower trace; note that our approach does not distinguish between proteins, lipids, amino acids, etc., all of which are capable to adsorb on particle surface). This finding is in agreement with a well-accepted concept that a biomolecular corona is immediately formed when nanoparticles come in contact with a biological milieu (even though the PEG functionalization is supposed to endow them with improved protein resistance [27,28]). For IONs coated by PEG and IL, the transformation into biomolecular conjugates takes obviously a longer time (see Fig. 2) and over the time of observation, the corona formation does likely not reach the state in which a stationary composition is attained.

Summarizing, by a smart coating, IONs can be enriched with different reactivity toward serum biomolecules and corona formation degree, with high relevance to the biomedical application. From the screening viewpoint, it is important that the developed assay is apt

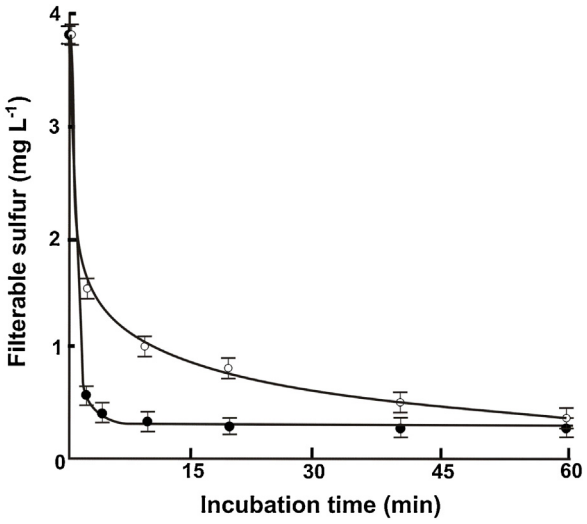

Fig. 2. A decline in the content of serum sulfur-containing compounds due to progressively enveloping the IONs covered with PEG (black circles) or PEG and IL (open circles).

to reliably monitor the transformations of IONs in real biosample irrespective of their kinetics.

4. Conclusion

The results have shown that sector-field ICP-MS offers a versatile tool of quantifying the target elements, iron and sulfur, in the bionano system under scrutiny (the limits of detection are 0.9 and 11.0 μg L^{-1}, respectively) and in combination with ultrafiltration, forms the basis of an adaptable screening platform to assess important pharmacological properties of engineered IONs. Focused on a series of novel IONs, we were able to evaluate relative stability, toxicity and reactivity in human serum and in this way, to portray them as candidates for further preclinical development. While a given set of characteristics may be not comprehensive, the lack of a single of them could impair the performance of IONs, making the difference between success and failure in clinics.

CRediT authorship contribution statement

Olga V. Kuznetsova: Formal analysis, Investigation. **Olga B. Mokhodoeva:** Conceptualization, Project administration. **Valeria V. Maksimova:** Formal analysis. **Rustam Kh. Dzhenloda:** Investigation, Methodology. **Maciej Jarosz:** Project administration, Writing - review & editing. **Valery M. Shkinev:** Resources, Supervision. **Andrei R. Timerbaev:** Conceptualization, Methodology, Project administration, Supervision, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113479.

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