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STABILITY OF NANOPARTICLE SUSPENSIONS IN DIFFERENT BIOLOGICALLY RELEVANT MEDIA

MOJCA PAVLIN[a*], VLADIMIR B. BREGAR[a,b]
[a]*Faculty of Electrical Engineering, University of Ljubljana, SI-1000 Ljubljana, Slovenia*
[b]*Kolektor Magma d.o.o., SI-1521 Ljubljana, Slovenia*

For application of nanoparticles (NPs) as delivery vectors for biomedical applications the size, charge, surface chemistry and aggregation of the particles are one of key parameters. We focused on characterization of maghemite and cobalt ferrite magnetic NPs by measuring size distribution and zeta potential in different culture media and PBS. We show that our NPs functionalized with PAA are relatively very stable also in different culture media, where level of aggregation depends on medium composition. Effect of divalent ions and serum presence on stability is specifically analysed and we present possible destabilization mechanisms. We show that stability of electrostatically stabilized suspensions is affected by the molar concentration and valence of destabilizing counterions like Na^+ or Mg^{2+} where effective surface charge of nanoparticles and thus repulsion force is screened by these counterions. In agreement with other papers we demonstrate significant effect of media composition (divalent ions, serum) and thus show the importance of NPs characterization under conditions that are representative of cell culture media or physiological conditions for understanding of NPs interaction with biological systems and for assessments of nanoparticle cytotoxicity. Further, by understanding the destabilization mechanisms one can anticipate effect in different media and to some degree predict behaviour of nanoparticle suspensions.

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1. Introduction

In biotechnology and biomedicine nanoparticles (NP) applications are becoming one of the key areas of research. Several therapeutics based on NPs have been successfully introduced for treatment of cancer, pain and infectious diseases as NPs offer the possibility of targeted delivery of drugs to specific locations, improved drugs' solubility and stability, and reduced side effects [1-3]. Important class of nanoparticles are NPs based on the magnetic materials, which can be manipulated by an external magnetic field. Magnetic nanoparticles are used in different promising biomedical applications, such as: cellular targeting, labelling and separation, tissue repair, drug delivery, contrast agents for MRI, hyperthermia and magnetofection [1-4]. In parallel, fast technological progress led to an ever increasing variety of products where different NPs are applied or produces. Therefore, the possible toxic effects of nanomaterials are now of great concern. Consequently, understanding of NPs characteristics in physiological environment is more and more important.

For application of nanoparticles as delivery vectors for drug targeting and similar biomedical applications the size, charge, surface chemistry and functionalization of the particles are particularly important since they strongly affect blood circulation time, aggregation, mobility and bioavailability of the particles within the body [5-7]. Magnetic nanoparticles have to have high

* Corresponding author: mojca.pavlin@fe.uni-lj.si

magnetization values, a size preferably smaller than 100 nm, and a narrow particle size distribution. Both, biomedical and biotechnological applications also need specific surface coating of the magnetic particles according to each specific application; but in general it has to be nontoxic and biocompatible and allow binding of drugs, proteins, enzymes, antibodies, or nucleotides. The surface modification by organic molecules has different tasks to fulfil: (i) stabilize the nanoparticles in a biological suspension with a pH around 7.4 and a high salt concentration, (ii) provide functional groups at the surface for further functionalization, and finally (iii) avoid immediate uptake by the reticuloendothelial system (RES) [8]. This functionalization additionally changes the properties of the nanoparticle and affects the bioactivity.

Important and challenging aspect of nanoparticle characterization is measurement under conditions that resemble in vitro or in vivo environment. One of the important parameters is stability and level of aggregation of nanoparticles in physiological conditions (e.g. plasma) or different media important for biotechnological applications (e.g. culture media). It was shown in several studies [9-17] that stability of NPs in different culture media can be severely reduced depending on ionic and protein composition consequently affecting NPs characteristic and functionality in in vitro and in in vivo applications. Since different parameters like size, charge and chemical properties determine quality and applicability of given nanoparticles characterization and analysis of NPs properties in different physiological conditions is crucial. For example, it was shown in several studies that stable nanoparticles in water or low-ionic buffer form large aggregates in physiological or similar conditions [9-17]. Different studies also demonstrated that characterization of nanoparticles in relevant media is necessary for evaluation of toxicity [12]. All these studies demonstrated that stability in given media is a complex combination of NPs surface properties, media compositions and nanoparticle concentrations, therefore characterization of NPs in physiologically relevant media is crucial for understanding of their interaction with biological systems.

In our study we focused on characterization of maghemite and cobalt ferrite magnetic nanoparticles. We characterize different sets of our magnetic NPs by measuring size distribution and zeta potential in different culture media and phosphate saline buffer (PBS). We show that our NPs functionalized with PAA are relatively very stable also in different culture media, where level of aggregation depends on medium composition. In addition, effect of divalent ions and serum presence on stability is specifically analysed and we present possible destabilization mechanisms. In agreement with other papers we demonstrate the importance of NPs characterization under conditions that are representative of cell culture media or physiological conditions for understanding of NPs interaction with biological systems and for assessments of nanoparticle cytotoxicity. Further, by understanding the destabilization mechanisms one can anticipate effect in different media and to some degree predict behaviour of nanoparticle suspensions.

2. Methods

In our study we examined the extent of the agglomeration for a set of magnetic nanoparticles, maghemite (MGH) core nanoparticles with citric acid surface coating [18,19], cobalt ferrite (CoF) nanoparticles [19,20] without any surface treatment, and cobalt ferrite nanoparticles with poly-acrylic acid (PAA) surface coating [21,22]. Suspension mass concentrations were determined with thermogravimetric method (moisture analyser MAC 50/1/NH, Radwag, Poland). Original dimensions of the nanoparticles and state in dry conditions were examined by transmission electron microscope ((JEM 2100) [19]. In suspensions dimensions and presence of agglomerates were examined by dynamic light scattering (DLS) (Nanosizer ZS, Malvern Instruments Ltd., UK) and confirmed for cobalt ferrite nanoparticles by magnetic susceptibility dynamic measurement [23,24] where we used custom set-up with the measuring coil connected to the impedance analyzer (Agilent 4294A). Agglomeration of nanoparticles and further destabilization of suspensions were determined by sudden increase of the average hydrodynamic diameter, as measured with DLS. Additionally, the agglomeration of suspensions was qualitatively observed also with visual observation of turbidity, where one can easily determine the onset of substantial agglomeration in dilute (transparent) samples.

Suspensions of nanoparticles without any surface treatment were prepared at two pH conditions, pH≈2 (adjusted with HNO_3) and pH≈12 (adjusted with tetramethyl-ammoniumhydroxyde -TMAOH). These suspensions were used only for titration with NaCl or $MgCl_2$ solutions since they are highly unstable in the physiological pH range of typical cell culture media. Suspensions of citric acid and PAA coated nanoparticles were prepared at pH=7.5 in DI water with concentration 1 wt% and addition of HCl for pH adjustment. A small volume of suspension, calculated to get desired final concentration, was added to different media like phosphate saline buffer (PBS), Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich Chemie GmbH, Germany) with 0,15mg/ml L-glutamine (Sigma Aldrich Chemie GmbH, Germany) and 0,1% gentamicine (PAA Laboratories, Austria), and Ham's tissue culture medium (HAM, Sigma Aldrich Chemie GmbH, Germany) with 0,5% L-glutamine (Sigma), 0,1% gentamicin (PAA Laboratories, Austria), and 0,1% penicillin (PAA Laboratories, Austria). In tests with serum we added to DMEM and HAM suspensions 10 vol% of fetal bovine serum (FBS, Sigma Aldrich Chemie GmbH, Germany) and to DI water suspensions 10-30 vol% FBS, with appropriate stock concentration to get the same suspension concentration of nanoparticles.

For test with electrolytes we diluted suspensions with DI water or media and titrated with NaCl, $MgCl_2$ or $CaCl_2$ solutions. Titrant solutions had molarity of 2,5M (NaCl) and 0,075M ($MgCl_2$) and total addition of titrant volume was about 10% of initial sample volume. Initial concentrations of nanoparticle suspensions were from 0.046% to 0,266 wt%, for titrated samples the end concentrations fell to about 10% lower values. For all suspensions we also measured nanoparticles' zeta potential (Nanosizer ZS, Malvern Instruments Ltd., UK) and determined isoelectric point (IEP) for each suspension. Approximate IEP can be clearly determined also by turbidity observation during titration. For all samples the incubation time before measurements was 5 minutes, which is in the range of often used incubation time in biotechnological applications. Results of measurement with DLS were obtained as an average of 30 repeated measurements and results of zeta potential measurements were obtained as an average of 20 repeated measurements.

3. Results

Initial physical characterisation showed that the magnetite/maghemite nanoparticles were magnetic [19] and showed saturation magnetization values of about M_0 = 40 Am^2/kg (MGH-CA), M_0 = 60 Am^2/kg (CoF), and 45 Am^2/kg (CoF-PAA). Characterisation with transmission electron microscope showed that the primary nanoparticles have core diameters of below 10 nm (MGH-CA) and about 10 nm (CoF), specific surface area measurements give approximately 1-2 nm larger diameters [19]. On the other hand, measurements of hydrodynamic diameter with DLS showed significantly larger measured diameters. These results are shown in figures 1-3 where measured size distribution by volume fraction are presented for MGH-CA, CoF and CoF-PAA nanoparticle suspensions. Volume fraction representation was selected over number fraction representation since volume fraction directly correlates to mass ratio of different fractions and is thus more descriptive for destabilization observation. All distributions are quite wide (polydisperse), however, one can observe an average hydrodynamic diameter of about 15 nm (MGH-CA) and 20 nm (CoF), indicating that the nanoparticles are composed of a few crystallites that form irreversible agglomerate with observed hydrodynamic diameter [19,22]. This is due to certain agglomeration during the preparation of particles and these agglomerates remain stable once the preparation procedure is completed. Nanoparticles coated with PAA are much larger (about 40 nm diameter), with some bimodal distribution. Due to the fixed CoF/PAA mass ratio we can conclude that the larger nanoparticles are made from several smaller agglomerates linked by PAA and not as single small agglomerate having a thick PAA layer.

Measurements of dynamic magnetic susceptibility, where again hydrodynamic diameter can be calculated from measured frequency response of magnetic susceptibility, gave approximately the same results (results not shown) as DLS for all CoF suspensions and thus further validated DLS measurements. The hydrodynamic radius measurements were approximately the same even in high-ionic strength suspensions where double layer of ions around nanoparticles/agglomerates is thin and thus difference between hydrodynamic and physical radius

is small [25]. Further evaluation of superparamagnetic state of the nanoparticles indicated that nanoparticles in suspensions form small agglomerates, in line with previous studies [26]. All initial suspensions were stable over extended time periods (min. few days) as evident from lack of sedimentation and repeated DLS measurements.

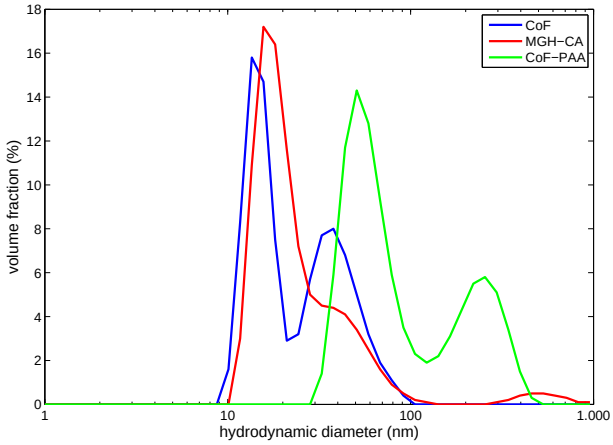

Fig. 1. Measured size distributions by volume for initial suspensions of MGH-CA, CoF and CoF-PAA nanoparticles.

In parallel we also measured zeta potential of all suspensions and determined approximate isoelectric points in order to determine range of stability for all suspensions. Surface coated nanoparticles (MGH-CA and CoF-PAA) were stable in the physiologically relevant pH range from 7 to 8,5. In order to validate the effect of surface charge we also used the same uncoated nanoparticles (CoF) at acidic (positive surface charge) and alkaline (negative surface charge) conditions.

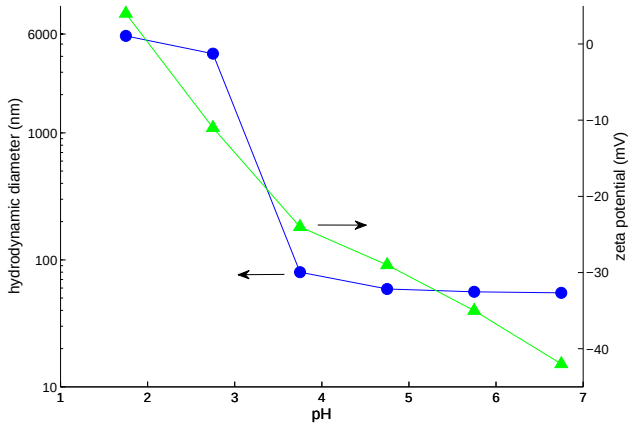

Fig. 2. Measured average hydrodynamic diameter (circles) and zeta potential (triangles) for CoF-PAA nanoparticle suspension. Suspension destabilization is clearly observed at zeta potential above -20mV.

Table 1: Measured zeta potential and approximate isoelectric point (IEP) for different suspensions in water.

	Zeta potential	approx. IEP
CoF pH=2,5	25	pH 5
CoF pH=12	-33	pH 5
CoF-PAA pH=7	-42	pH 2
MGH-CA pH=7	-35	pH 4

3.1 Effect of ionic strength on stability of nanoparticles

For evaluating the effect of different ions on suspension destabilization we titrated the suspensions with different salt suspensions (NaCl, $MgCl_2$, $CaCl_2$). In Fig. 3 are shown measurements of hydrodynamic diameter as a function of NaCl molar concentration for CoF and CoF-PAA suspensions. We can observe stability of CoF-PAA suspensions at much higher ionic strengths compared to CoF suspensions, which can be explained by additional electrosteric [15,27] stabilization of PAA polymer and also by much larger initial surface charge of CoF-PAA, as evident from Table 1. For MGH-CA nanoparticle suspensions we observed high stability although the initial surface charge was more close to uncoated CoF nanoparticles, which we explain by some added steric stability despite quite short length of citric acid and different interaction of CA with water medium. The optical observation of suspension stability is shown in Fig. 4 where there can be clearly seen that onset of turbidity (agglomeration), which is directly related to marked increase of the hydrodynamic diameter, leads to sedimentation in relatively short time span.

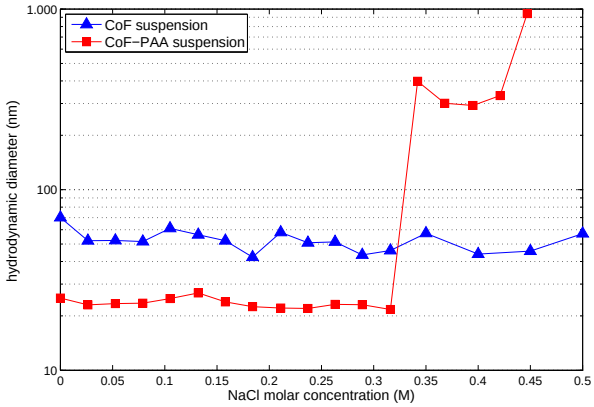

Fig. 3. Stability of alkali CoF and CoF-PAA suspensions at different NaCl molar concentrations. One can observe much better stability for surface coated nanoparticles. Suspension concentration is 0,266 wt%.

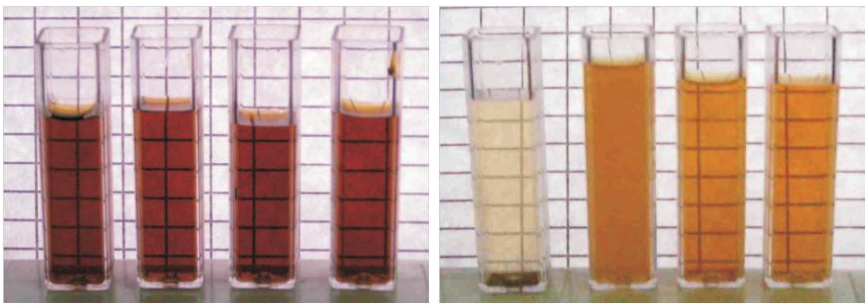

Fig. 4: (a) Samples of CoF-PAA suspension with different NaCl concentrations (from right: 0,5 wt%, 1wt%, 2wt%, 3 wt%). Concentration of nanoparticles in the suspension was 0,266 wt%.(b) Stability and aging of MGH-CA suspension (concentration 0,1 wt%) with different NaCl concentrations (from right: 1 wt%, 2wt%, 3wt%, 3wt % after 4 hours).

The mechanism of ionic strength effect on the stability of electrostatically stabilized suspensions is obviously a decrease of the effective surface potential due to formation of counter ion layer [27,28], as seen from Table 2. However, there is an additional effect of ion valence that is much more pronounced than the decrease of effective surface potential. This effect is presented in Fig. 5 where results of titration with $MgCl_2$ on the hydrodynamic diameter are shown. Tested suspensions had either positive surface charge (acidic conditions) or negative surface charge (alkaline conditions) and evident is significant difference of the Mg^{2+} molar concentration on the stability (hydrodynamic diameter), with alkaline suspension being destabilized by molar concentrations below 1mM. Further examples of Mg^{2+} destabilization effect are shown in Fig. 6 for CoF-PAA and MGH-CA suspensions. In Table 2 are presented also measured zeta potentials for CoF-PAA suspensions with different ionic strengths and ions in suspensions.

Table 2: Measured zeta potential for CoF-PAA and MGH-CA suspensions at different electrolyte molar concentrations. In all cases suspension concentrations were 0,133 wt%.

	Zeta potential	Hydrodynamic diameter
Co-ferrite+PAA (pH=8)	-59mV	60 nm
Co-ferrite+PAA +NaCl (0,25M)	-33mV	71 nm
Co-ferrite+PAA +NaCl (0,5M)	-24mV	49nm+190nm+1.2µm (aggl.)
Co-ferrite+PAA +MgCl2 (0,004M)	-50mV	65 nm
Co-ferrite+PAA +MgCl2 (0,008M)	-32mV	80nm+260nm (aggl.)
MGH-CA	-33mV	30nm
MGH-CA +NaCl (0,5M)	-12mV	15nm+35nm (aggl.)

As expected, the Mg^{2+} ions have effect only on nanoparticles with the negative surface charge as evident from Fig.5, where alkaline suspension with negative surface charge destabilizes at very low molar concentrations of $MgCl_2$. In contrast, for acidic suspension we observe destabilization only when Cl^- molar concentration reaches destabilization level similar to the one observed with NaCl in Fig.3. From Fig. 6 we observe that both types of surface coated nanoparticles (CoF-PAA and MGH-CA) have also for Mg^{2+} ions higher destabilization levels than naked nanoparticles, which can be again attributed in large part to both higher surface charge and additional steric stabilization.

Importantly, by comparing NaCl and $MgCl_2$ titration test a notable difference in destabilization levels is noted. The nanoparticle suspensions are stable to over 0,3M NaCl molar concentrations whereas the suspensions destabilize at molar concentrations of 1-20 mM $MgCl_2$. Similar results are obtained also with $CaCl_2$ salts, which confirms that the observed effect is indeed the effect of ion valence and not specifically Mg^{2+} ions.

According to the DLVO theory of electrostatic stabilization the main parameter for the effect of ions on suspension destabilization is the ionic strength of the suspension [9,17,27-30], which affects electrostatic double layer and electrostatic repulsive potential. The effect of ions on the zeta potential of suspensions is shown in Table 2. However, the ratio of ionic strength *I* for $MgCl_2$ and NaCl calculated from equation (1) [IUPAC]

$$I = \frac{1}{2}\sum_i c_i z_i^2 \qquad (1)$$

where c_i denotes molar concentration of *i*-th ion and z_i denotes charge of *i*-th ion, is only 3:1 and cannot explain the observed destabilization level ratio of two orders of magnitude. Also the measured zeta potentials are similar for stable NaCl/CoF-PAA and destabilized $MgCl_2$/CoF-PAA suspensions and thus by itself cannot explain destabilization.

On the other hand, it is known that divalent and multivalent ions can exhibit increased surface affinity [9,27,30] and behave also as a bridge between two (opposite to ion) same-charge nanoparticles [9,11,16,27-31]. This bridging could thus directly assist in agglomeration of two nanoparticles (as in case of CoF suspensions) and additionally compact the surface polymer (as in case of CoF-PAA suspensions). The latter effectively reduces surface charge of nanoparticle coating and also reduces steric stabilization.

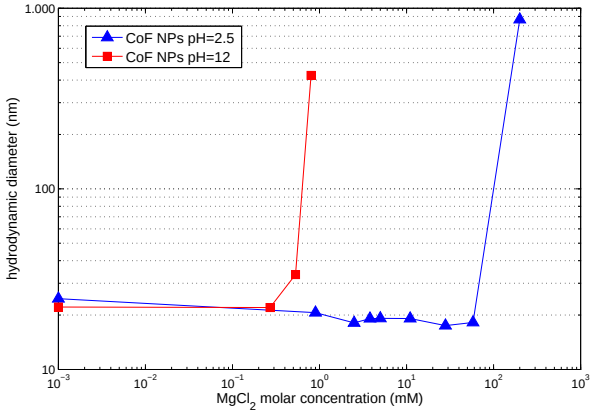

Fig. 5. Effect of Mg^{2+} molar concentration on alkaline and acidic CoF suspensions. Evident is significant effect on negative surface charge nanoparticles (alkaline suspensions) while for acidic suspensions destabilization level due to Cl^- are comparable to values from Fig.3. Nanoparticle concentration for both suspensions was 0,133 wt%.

Our results are in agreement with other reports [11] where it was shown that divalent ions are much more effective in destabilizing silica nanoparticles compared to monovalent ions. We confirmed these findings also for surface coated nanoparticles where some steric stabilization with charged polymers is also present. Although in literature a good agreement between ionic strength and destabilization level is observed [17], the particles in this study were sterically stabilized with uncharged polymers. In contrast, with surface charged nanoparticles we observed much stronger effect of divalent ions, as also observed in [11], and thus identified ionic strength as insufficient parameter for evaluation of the general suspension stability.

3.2 Effect of nanoparticle concentration on stability

With our experiments we observed also an unexpected effect of nanoparticle concentration in suspension on destabilization levels of molar concentrations of $MgCl_2$. As evident from Figs. 6 and 7 we observed with decreasing suspension concentrations destabilization at significantly lower ion molar concentrations. The observation was confirmed with turbidity measurements shown in Fig.8. This effect is somewhat unexpected since the probability of nanoparticle collisions increases with concentration square [30] and thus one would expect higher stability at lower concentrations [9,11]. On the other hand, reduced suspension concentration increases the ratio between number of divalent ions and single nanoparticle. In combination with the bridging effect of divalent ions this could lead to increased destabilization. Observed roughly linear dependence of destabilization molar concentration level of divalent ions on nanoparticles concentration indicates that the relevant destabilization parameter is the ratio of divalent ions to the number of nanoparticles that affects bridging. Both bridging and collision dependence should lead to a destabilization level minimum and with further study we will try to determine the concentration level where increased stability is again observed. This will help evaluate optimum concentrations of suspensions in real applications.

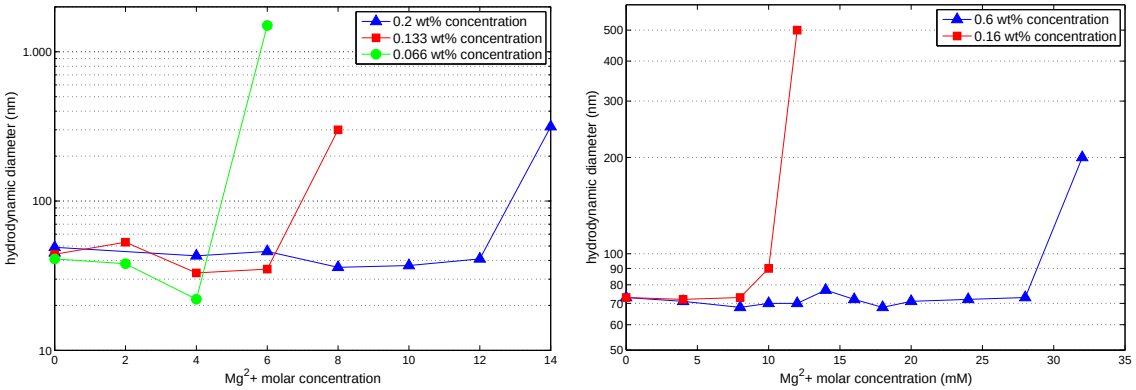

Fig. 6. Measurements of average hydrodynamic diameter as a function of Mg^{2+} molar concentration for suspensions with different concentrations. Upper graph shows results for CoF-PAA suspensions, lower graph shows results for MGH-CA suspensions.

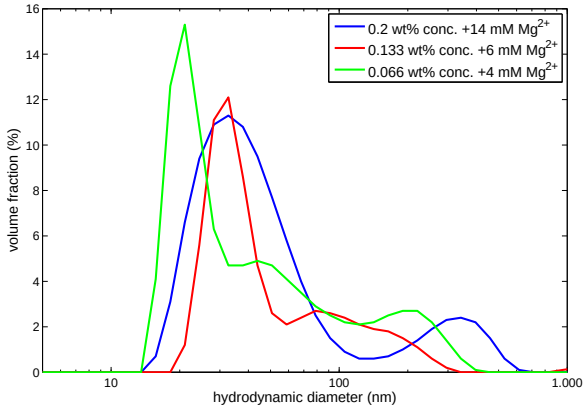

Fig. 7. Measured distribution by hydrodynamic diameter for CoF-PAA suspensions having different nanoparticle concentrations. Suspensions are measured at boundary Mg^{2+} molar concentrations with onset of destabilization.

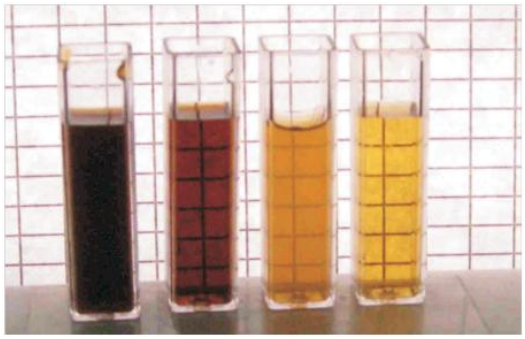

Fig. 8. Comparison of visual agglomeration for different concentrations of nanoparticles. Two samples on the left have 3x higher concentration (0,2 wt%) than samples on right (0,066 wt%). Clear samples are initial stable suspensions; turbid samples have 14 mM (left) and 6 mM (right) Mg^{2+} ion concentration.

3.3 Stability in physiological media

Further experiments were designed to evaluate the effect of ionic strength and presence of divalent ions in physiological media on the stability of nanoparticles, with implications for use of nanoparticles in biomedical and biotechnological applications. We used three media that are common in in vitro: two culture media (DMEM, HAM) and medium that is often used for analysis in vitro (PBS). All three media have significant NaCl molar concentration, but the first two have also non-negligible molar concentrations of divalent Ca^{2+} and Mg^{2+} ions and to all media fetal bovine serum (FBS) is usually added for cell culturing or incubation. Stability in the physiological media could be affected by a quite large NaCl molar concentration and presence of divalent ions; however, the molar concentrations are notably smaller than the destabilization levels observed in our previous experiments. Here a relatively short incubation time of suspensions (time delay between preparation and characterization) is quite relevant as with longer time scale (e.g. longer incubation in vitro) we can expect increasingly stronger destabilization [10,17].

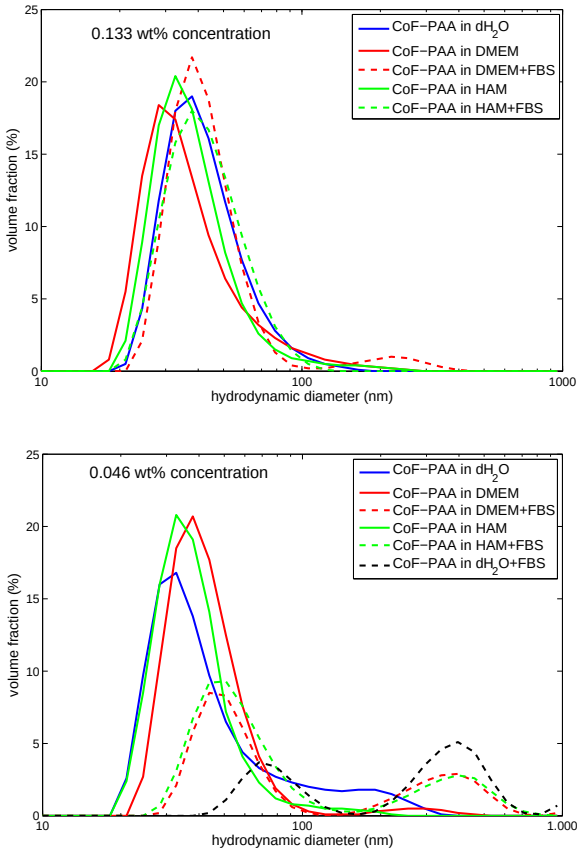

Fig. 9: Measured hydrodynamic diameter distribution by volume for CoF-PAA suspensions in different media. All results for PBS medium are almost identical to the results for water-based suspensions and are not shown.

Results for both DMEM and HAM media with and without added serum are presented in Fig. 9, whereas PBS medium showed similar results as water-based suspensions and the results are not shown. In the applications the used nanoparticle concentrations are usually low and given the results of our experiments on the importance of concentration we evaluated stability at two concentrations, 0,133 wt% and 0,046 wt%. For the higher concentration we observed almost no effect of culture media on stability, regardless of presence of serum, in agreement with literature reports where PAA coated nanoparticles exhibit very good stability [15]. On the other hand, for lower concentration there was significant effect of serum with immediate destabilization at the physiological serum concentrations (10 vol%). This demonstrates that stability in physiological conditions is a complex function of nanoparticles surface characteristics and components of surrounding medium in agreement with other reports. For example, aggregation of nanoparticles in

in vivo conditions is well known problem [8,10,33,34], and is a consequence of proteins and peptides adsorption to the charged nanoparticle surface. On the other hand there are several studies [9,13,16] identifying the increased stability of nanoparticles (e.g. TiO2 and similar technologically relevant nanoparticles) by the addition of the serum in the medium, with the stabilization mechanism being again opsonization of the nanoparticle surface with proteins and peptides, which form stabilizing protein corona.

Measurements of water-based suspensions with added serum showed similar size distribution and destabilization of nanoparticle suspensions at higher serum concentrations (30 vol% and above), but stable suspension at usual concentration (10 vol%). This suggests that even if ions alone cannot destabilize the suspension, the serum presence acts as the additional factor in media and can push the suspension over the destabilization level. Reduction of the effective surface charge is evident also from Table 3 where measurements of zeta potential for different suspensions are presented. One can observe additive effect of serum and ionic strength in DMEM and HAM on zeta potential; however, again there is inconsistency in similar zeta potential level for (stable) suspensions in DMEM and HAM media without serum and (unstable) water-based suspensions with 30 vol% serum added. This could be explained both with additional surface coating by serum proteins (opsonization) [10,14,16,33,34], and with similar interaction of serum proteins with nanoparticle surface as in case of monovalent ions. Due to relatively high serum concentration and observed destabilization levels we exclude any bridging effect as although protein charge can be significant, there is also substantial charge distribution (e.g. [35]), with protein size adding to the steric repulsion.

Table 3: Measurements of zeta potential for suspensions in different media.

Suspension (0,046 wt% CoF-PAA)	***Zeta potential***
CoF-PAA +DMEM	-32 mV
CoF-PAA +DMEM+serum (10 vol%)	-15 mV
CoF-PAA +HAM	-26 mV
CoF-PAA +HAM+serum (10 vol%)	-17 mV
CoF-PAA +serum (10 vol%)	-35 mV
CoF-PAA +serum (20 vol%)	-30 mV
CoF-PAA +serum (30 vol%)	-27 mV

The difference between our results on media stability, similar results from other studies [8,10,14,15,33,34], and the observed results from several studies where increased stabilization with addition of serum was observed [9,13] can be explained with two important differences. The surface charge of the nanoparticles used in our study is negative, with stable biopolymer layer on surface, whereas nanoparticles in mentioned studies have positive surface charge, without any surface coating, and were distributed in relatively large agglomerates.

Nevertheless, all these results show the important effect of surface conditions on interactions with the other compounds in the medium and the necessity of characterization in the actual environment of application. It also shows that simple nanoparticle suspensions could be very unstable in body fluids in in vivo conditions and further (steric) stabilization is usually necessary. For the example of latter, in one study [10] authors observed that proteins formed permanent corona on nanoparticle surface in RPMI medium and are more abundantly internalized in cells as compared to DMEM medium, overall exerting higher cytotoxic effects. Altogether, these results show that before cellular experiments, a detailed understanding of the effects of cell culture media on nanoparticle suspensions is crucial both, for optimized biomedical applications and for standardized nanotoxicology tests.

4. Conclusions

Stability of suspension of nanoparticles is primary dependent on the surface condition of the nanoparticles, where value of the surface charge define strength of electrostatic stabilization,

sign of the surface charge define susceptibility to different ions, and coating with polymer or similar molecules add steric stabilization. For both in vitro and in vivo applications the sign of nanoparticle surface charge is selected according to required functionality of nanoparticles and is usually not freely selected; therefore it is important to understand what types of ions affect the stability in biologically relevant media and physiological conditions.

Stability of electrostatically stabilized suspensions in bioapplications is usually affected by the molar concentration and ionic strength of destabilizing counterions like Na^+ or Mg^{2+} where effective surface charge of nanoparticles and thus repulsion force is screened by these counterions; yet valence of ions like Mg^{2+} turned out to be extremely important. Our results show that divalent ions are much more potent flocculants than monovalent ions, with the destabilization molar concentration for divalent ions (e.g. Mg^{2+}, Ca^{2+}) a few orders of magnitude smaller than for monovalent ions (e.g. Na^+, K^+, Cl^-). Further, the ionic strength alone despite included charge valence cannot predict observed destabilization molar concentration levels of divalent ions and is thus not a suitable general parameter for medium destabilization potential evaluation.

The divalent ion destabilization we attribute to bridging effect where oppositely charged divalent ion forms a link between two nanoparticles with the same sign of surface charge. For the nanoparticle suspensions that we used destabilization molar concentration levels were significantly higher than the usual molar concentrations of positive (like Na, K, Ca, Mg) or negative (like Cl, SO_4, H_2PO_4) single counterions, which are most common in biologically relevant media.

For divalent ions we also observed the dependence of nanoparticle concentration in suspension, but contrary to expectations we observed decreased stability with decreasing concentration. This effect can be explained with ratio of divalent ions to number of nanoparticles being the relevant parameter for destabilization. Due to the collision dependence on nanoparticles concentration we expect under some limit level again increased stability with decreasing suspension concentration. The concentration stability dependence could be important for the biotechnological and biomedical applications since a wide range of nanoparticle concentrations are used, e.g. relatively low concentration in vivo and relatively high concentrations in vitro.

Furthermore, our results also confirmed the effect of proteins on the stability of nanoparticles. Opsonization, formation of protein corona, of nanoparticles further destabilized the nanoparticle suspensions and addition with the effect of ions led to a certain degree of flocculation and sedimentation of otherwise very stable CoF-PAA suspension. The destabilization mechanism is reduction of the effective surface charge, however, both reduction of the effective surface charge and bridging effect with divalent ions are to some degree accumulative and the combination of ions and peptides in media could well lead to destabilization. This is very relevant for biological applications since culture media or plasma serum contain not only simple ions but also peptides that can exhibit negative or positive charged groups at physiological conditions and form transient or even permanent surface corona. Even more importantly for biomedical and biotechnological applications, the surface coating with proteins could affect the interaction with membranes and cells.

Altogether, our results stress importance for characterization in relevant physiological conditions in order to analyse NPs intracellular fate and cytotoxicity. Further, by understanding the destabilization mechanisms one can anticipate effect in different media and to some degree predict behaviour of nanoparticle suspensions in given application. Specifically, when using one type of NPs on different cell lines the dynamics and amount of internalization as well as intracellular fate [22] depend not only on specific cell line characteristics or NPs properties but also on mutual interaction of NPs with culture medium. For in vivo applications it also clear that stability of nanoparticles can be severely reduced in plasma serum consequently affecting overall bioavailability since large aggregation reduces mobility, or can be potentially toxic. All these results and findings improve the understanding of the nanoparticle drug delivery systems at relevant biological conditions and can enhance the efficiency of the applications.

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