SYSTEMATIC INVESTIGATION OF INTERACTIONS OF BULK NANOBUBBLES WITH SOFT MATTER

Minmin Zhang

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NANOBUBBLES WITH SOFT MATTER**

Minmin Zhang

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,

Dedicated to my family.

献给我最亲爱的家人

"Life is like riding a bicycle.
To keep your balance, you must keep moving."

– Albert Einstein

"行到水穷处，坐看云起时。"

– 王维

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INTRODUCTION

This chapter gives a general introduction to nanobubbles, particularly bulk nanobubbles, and a brief description of their characteristics. A summary of generating and characterizing methods for bulk nanobubbles is presented, their hypothesized stabilization mechanisms are briefly discussed, and a brief review on the experimental studies of applications with bulk nanobubbles is given. The chapter ends with an overview of the aims and the outline of this thesis.

1.1 Bulk nanobubbles

Nanobubbles are nanoscopic gas bubbles that form both on submerged surfaces and in bulk solutions. They are known as “surface nanobubbles” (height 10–100 nm, radius of contact line 50–500 nm) and “bulk nanobubbles,” (gas-filled spherical in bulk, diameter of less than 1 μm), respectively.^{1–16} They were first speculated to exist in 1994, based on the step-wise force discontinuity features in surface force measurements observed between two hydrophobic surfaces.¹⁷ In 2000, the first atomic force microscopy (AFM) image of surface nanobubble was obtained by Ishida *et al.*¹⁸ and Lou *et al.*¹⁹ Some landmark results in this field are summarized in Figure 1.1. Nanobubbles have attracted attention in the scientific world mainly driven by their longevity, as a classical estimate by Epstein and Plesset predicted that they should dissolve within a diffusion timescale $\tau_{\text{dis}} \sim R^2/D \sim 10 \mu\text{s}$ due to the high Laplace pressure inside the nanoscopic objects,^{20, 21} based on a typical bubble radius $R \sim 100 \text{ nm}$ and gas diffusion coefficient $D \sim 1 \times 10^{-9} \text{ m}^2/\text{s}$. Instead, nanobubbles are observed to survive for days.^{22, 23}

1.1.1 Terminology

Here we concentrate primarily on bulk nanobubbles, focusing on their properties and experimental studies thereof. To avoid confusion between surface nanobubbles and bulk nanobubbles, we use *surface* when discussing aspects specific to surface nanobubbles and *bulk* nanobubble when demonstrating points related to bulk nanobubbles. If we remove the prefix, simply calling it *nanobubble* or *bubble*, this also means that we are discussing bulk bubbles. This convention holds throughout the entire thesis.

Figure 1.1: Timeline of some landmark publications in the field of nanobubbles. Contributions to bulk nanobubbles are highlighted in bold. **1950: Epstein-Plesset theory is used to predict the lifetime of a single bubble. According to this theory, a nanobubble in a saturated solution should dissolve in microseconds.**²⁰ 1994: Surface nanobubbles were proposed to account for very long range attractive forces between two hydrophobic surfaces.¹⁷ 1997: The role of surface nanobubbles in the long range attractive forces between hydrophobic surfaces claimed earlier¹⁷ was dismissed by more extended theoretical arguments.²¹ 2000: The first images of surface nanobubbles using atomic force microscopy (AFM) were published.^{18, 19} **2003: The application of bulk nanobubbles as ultrasound contrast agents was reported.**²⁴ 2006: The effect of salt and surfactants on the shape and size of surface nanobubbles was found to be negligible, thus indicating that the stabilization of surface nanobubbles was unlikely to be due to contaminants.²² **2010: Replicas of bulk nanobubbles were imaged by cryo-scanning electron microscopy.**²⁵ **2014: The relative mass density of nanoparticles compared to the solvent was measured by a microresonator. The results indicate that the particles are gaseous.**²⁶ 2015: Seo *et al.* directly showed that surface nanobubbles are gaseous using fluorescence microscopy coincided with other imaging techniques.²⁷ Reprinted and adapted with permission from M. Alheshibri *et al.*; *Langmuir*, **2016**, 32, 11086–11100. Copyright © 2016 American Chemical Society.

1.1.2 Generation and characterization of bulk nanobubbles

A prerequisite for investigating bulk nanobubbles as well as exploring their potential applications is to develop a protocol for generating them.^{14, 28–30} Water electrolysis, solvent exchange, temperature increase and ultrasonication can all trigger the formation of bulk nanobubbles. Essentially any mechanism that leads to gas supersaturation drives their formation.^{31–34} Particularly vital are characterization techniques that are able to discern whether the bubbles actually consist of gas.

Solvent exchange and temperature increase method

The solvent (ethanol–water) exchange method was first used to produce surface nanobubbles,^{7, 23} and was later found to also generate bulk nanobubbles.³² The standard protocol of the solvent exchange method is to first inject ethanol in the fluid cell, next replace the ethanol with water. As ethanol is miscible with water and has a higher solubility of atmospheric gases under atmospheric pressure and at room temperature, a local supersaturation of gas in the fluid cell is created during the second step, and, subsequently, bulk bubbles are formed.³² An extension of the solvent exchange method is the temperature increase method.³³ Nanobubbles can be generated under gas supersaturated solution induced by temperature increase. Najafi *et al.* generated nanobubbles in gas-saturated solution by manipulating the temperature via a sudden increase.³³ The solubility equilibrium is disrupted by the sudden change in the temperature and results in nucleation of bulk nanobubbles. Abrupt increase in the temperature of gas-saturated solution facilitates the generation of bulk nanobubbles and thermal treatment also provides adequate energy for the development of the gas/water interface.^{35, 36} A nanobubble size of 290 nm was characterized by a dynamic light scattering (DLS) method. These authors show that the zeta potential of the nanobubbles generated in 10 mM NaCl solution is negatively charged with an isoelectric

point of pH 2.2–2.4, which is consistent with other reports.^{37, 38}

Water electrolysis

Water electrolysis has also been used to generate bulk nanobubbles.^{31, 39–45} Water electrolysis can generate solutions supersaturated with oxygen gas at the anode and hydrogen gas at the cathode. Subsequently, stable colloids are observed in the supersaturated solution. Takenouchi *et al.* used platinum-coated titanium electrodes to electrolyze water with dilute potassium carbonate as electrolyte, resulting in an alkaline electrolyzed water with hydrogen nanobubbles at the cathode. DLS revealed that in a closed system hydrogen nanobubbles have a diameter of about 128 nm even 24 h after formation, whereas in an open system Ostwald ripening was observed. However, in both systems, after a week, a few hydrogen nanobubbles smaller than 300 nm diameter can still be observed.

Ultrasonication

Another way to generate bulk nanobubbles is by ultrasonication.^{34, 46, 47} The earliest report that suggested the generation of bulk nanobubbles could be traced back to 1962. Sette and Wanderlingh suggested that nucleation in ultrasonic cavitation is influenced by cosmic radiation. They imply that the generation of bulk gaseous bubbles at low concentrations may be connected to heating and radiolytic effects. This would create small overheated H₂ and O₂ oversaturated regions along the path of radiation, inside which bulk bubbles may be generated. In addition, the generated bulk bubbles seem to be stabilized by a process associated with impurities.⁴⁷ Kim and his colleagues generated bulk nanobubbles by ultrasonication with a two-sided 5 mm × 10 mm palladium coated electrode at a frequency of 42 kHz. DLS and electrophoresis give the bubbles an average size of 300 to 500 nm and negative zeta potentials at all pH, respectively.³⁴ Yet, the mechanism of nanobubble

formation with ultrasonic energy has not been fully understood.⁴⁶

Other characterization techniques

Much of the success in observing bulk nanobubbles is due to the employment of DLS^{48, 49} and electrophoresis^{28, 46} measurements. However, a number of other techniques have also contributed substantially, such as nanoparticle tracking analysis,^{32, 39} replica scanning (transmission) electron microscopic imaging,^{25, 50} and resonant mass measurement etc.²⁶ We briefly describe each technique in the following discussion.

Nanoparticle tracking analysis Nanoparticle tracking analysis (NTA), a novel method of visualizing bulk nanobubbles was developed and substantially employed in recent years.^{32, 39} NTA tracks the motion of a number of individual nanoparticles, then extracts diffusion coefficients and sizes for each individual nanoparticle. Compared with DLS, NTA thus has the advantage of being able to collect and analyze size information from individual nanoparticles, rather than averaging the ensemble. By measuring the exact number of particles within a given volume, the number density (concentration) can be easily calculated by NTA. Qui *et al.* studied the formation and stability of bulk nanobubbles by using NTA.³² They found a concentration of $\sim 10^8/\text{mL}$ which was about five times higher than for solutions that were degassed.

Replica scanning (transmission) electron microscopic imaging

Replica scanning electron microscopic (SEM) imaging method is another reliable method for observing the sizes of nanobubbles in liquid.²⁵ In this method, the internal and interfacial structure of bulk nanobubbles are preserved by rapid cryogenically freezing a small drop of aqueous solution in liquid 2-methylbutane (at 113 K). The freezing structure is then cracked to disclose the sectional view. Subsequently, the plasma film-deposition method is applied to form a hydrocarbon thin-film of replica on the cross section. The

Figure 1.2: (a) SEM image of freeze-fractured replica of N₂ bulk nanobubbles and (b) a magnified image of a N₂ bubble with a diameter of 100 nm. Reprinted and adapted with permission from K. Ohgaki *et al.*; *Chemical Engineering Science*, **2010**, 65, 1296–1300. Copyright © 2010 Elsevier Ltd.

ice freezing structure is then removed by heating and the replica is viewed by SEM. As shown in Figure 1.2, Ohgaki *et al.* measured the average size of the bulk nanobubbles of 100 nm in diameter.²⁵ Uchida *et al.* extended this method by using transmission electron microscope (TEM) to view the replica of oxygen nanobubbles. Figure 1.3 shows that nanobubbles in aqueous solution are spherical or oval and their size distribution from TEM images is close to the results from DLS.⁵⁰

Resonant mass measurement Perhaps the most convincing method to prove that bulk nanobubbles contain gas is the resonant mass measurement conducted by Kobayashi *et al.*²⁶ The resonant mass measurement is a particle size and mass measurement which detects mass density of individual nanoparticles relative to the solvent as they pass through a microresonator. When nanoparticles flow through the resonator, the resonant frequency changes, shifting toward high frequencies for particles less denser than the solvent and shifting toward low frequencies for particles denser than

the solvent. Thus, the resonant mass measurement method is capable of distinguishing positively buoyant particles (bubbles) from negatively buoyant particles (dust particles) by detecting the changes in the resonant frequency. By using this method, the authors showed that bulk nanobubbles have a positive buoyancy with a modal diameter of 112 nm which is again consistent with that determined by DLS and NTA.²⁶

The vast amount of experimental data with distinct generating and characterizing methods presented above clearly demonstrates that bulk nanobubble do exist. But many questions still remain unanswered on the origin of their stability, a topic we turn to next.

1.1.3 Stability of bulk nanobubbles

As discussed, based on classic nucleation and growth theories, bulk nanobubbles should be out of equilibrium, either dissolving or growing depending on the saturation level of the solution near the gas/liquid interface. Yet, they are found to be stable for hours or even days.^{6, 9, 10, 51}

Negligible buoyancy force and electrostatic double layer force

Negligible buoyancy force First, because nanobubbles are small, the buoyancy force on them is also small and can be neglected compared with thermal energy.⁸ The rise time of a spherical bubble in a quiescent liquid can be estimated. The terminal rise velocity is obtained when a nanobubble's buoyancy force is balanced by the drag. For a no-slip boundary condition,⁵² the terminal rise velocity v of a bubble with radius r is then given by Stokes Law,

$$v = \frac{2r^2\Delta\rho g}{9\eta}, \quad (1.1)$$

Figure 1.3: TEM images of freeze-fractured replica of pure O_2 bulk nanobubbles in water. Spherical or oval nanobubbles with a diameter of (a) 500 nm and (b) 200 nm. (c) Blank: the replica sample of pure water without aeration. Each scale bar represents 500 nm. Reprinted and adapted with permission from T. Uchida *et al.*; *Nanoscale Research Letters*, **2011**, 6, 295. Copyright © 2011 licensee Springer.

where $\Delta\rho$ is the difference in density between the bubble and the liquid, g is the acceleration of gravity, and η is the liquid viscosity. A bubble of radius 100 nm has a terminal rise velocity of ~ 20 nm/s. Suppose that the container is a cuvette with a height of $h = 1$ cm, the rising time of the nanobubble is around

5 days in aqueous solution. This means that for sufficiently small bubbles, Brownian motion effectively competes with buoyancy over the experimental time scale.

Electrostatic double layer repulsion Next, the electrostatic double layer repulsion also acts to stabilize nanobubbles.^{28, 51, 53} The electrostatic double layer repulsion exists due to the negatively charged gas/liquid interface.^{53–58} It has been known since the 19th century that air bubbles at neutral pH water bear a spontaneous negative surface charge based on the observations that they migrate toward the positive electrode in electrophoretic measurements.⁵⁹ McTaggart further confirmed the results of electrophoresis by using a cylindrical rotating cell to offset the buoyancy of the bubble and speculated that the negative surface charge is due to the selective adsorption of hydroxide ions.⁶⁰ Observations of pH dependence of zeta potential of air bubbles confirming this hypothesis were then reported upon.^{54, 55, 58, 61, 62} Takahashi *et al.* measured a zeta potential of -100 mV at pH 10, which decreased to -35 mV when decreasing pH to 5.8. The measurement of pH dependence of zeta potentials gives an isoelectric point at pH 3–4.⁶² Creux *et al.* found that, in neutral water, the zeta potentials of gas bubbles formed by four different types of gases (nitrogen, oxygen, helium and industrial air) are the same (-63 ± 2 mV) within experimental error.⁵⁵ Furthermore, the effect of increasing electrolyte concentration on the zeta potentials was investigated as shown in Figure 1.4. The zeta potential is reduced (becomes less negative) as the sodium halide salt concentration increases above 10^{-5} M due to double layer compression. The negative zeta potential increases (becomes more negative) upon increasing the NaOH concentration, strongly suggesting the specific adsorption of hydroxide ion at the interface.⁵⁵

To further confirm the above view, we measured the pH dependence of the zeta potentials in our nanobubble solutions at a NaCl concentration of 10 mM, as discussed in detail in Chapter 3. The results are shown in

Figure 1.5. As expected, a negative surface charge on gas bubbles in aqueous solutions is observed, consistent with the experimental observations reported previously.^{28, 53, 55, 62}

Figure 1.4: Zeta potential of nanobubble solution as a function of (a) salt concentration (squares: NaI; discs: NaBr; diamonds: NaCl) and (b) alkali concentration (diamonds: NaOH; squares: LiOH; triangles: CsOH). Reprinted and adapted with permission from P. Creux *et al.*; *The Journal of Physical Chemistry C*, **2007**, 111, 3753–3755. Copyright © 2007 American Chemical Society.

Figure 1.5: Zeta potential of nanobubble solutions as a function of pH in 10 mM NaCl at 25 °C.

These observations lead to the unambiguous conclusion that the air/water interface of bubbles is negatively charged. Strong adsorption of hydroxide ions probably accounts for the negative charge. This negative surface charge of the hydroxide is balanced by protons or salt in the diffuse layer to satisfy electroneutrality. In this sense, water itself is acting to stabilize the air bubbles against coalescence.⁵⁴ A number of speculations have been proposed attempting to model the gas/water interface at the molecular scale, but so far none seems to provide a convincing explanation, some even contradicting the experimental evidence that the surface of neat water is basic.^{63–70}

Reduction of surface tension

Secondly, nanobubble stability can be increased by a reduction in Laplace pressure arising from the decrease of surface tension at the gas/water interface. What are possible reasons for a reduction of the surface tension of nanobubbles?

One possibility is impurity. Impurities from improper sample preparation, sloppy experimental procedures or organic impurities in the solvent employed, will reduce the surface tension of gas/water interface.^{71–77} The theory proposed for the stability of surface nanobubbles by Ducker *et al.* can also be applied to bulk nanobubbles. They assume that a layer of water insoluble organic contaminants coats at the gas/water interface, which decreases the surface tension and obstructs the diffusion of gases from nanobubbles, thus further increasing the nanobubble lifetime.⁷² It is known that ultrapure air–water interfaces are vulnerable to airborne contaminant molecule and contaminants could also arise from packaging materials of experimental tools, e.g. AFM cantilever, plastic syringe etc.^{12, 74, 76–78} This interpretation is further supported by the removal of nanobubbles using sodium dodecyl sulfate (SDS) micelles, which presumably solubilize the water-insoluble contaminants from the gas/water interface resulting in nanobubble

removal.⁷²

Another possibility that has been put forward is that of adsorption of ions at gas/water interface. The presence of ionic components due to thermal electrolytic dissociation as well as some level of carbonic acid anions at the gas/water interface could also reduce surface tension and help stabilize the nanobubbles.^{51, 56, 79–83} Bunkin *et al.* proposed a quantitative model that gas bubbles are stabilized by adsorbed ions, which charge the bubble surface and are screened by a cloud of less adsorbing counter-ions.^{51, 82}

Supersaturation

A stable colloidal bubble solution can be achieved when bubbles are generated under supersaturated solution.^{9, 84–87} This may be the case when large number of bubbles are generated. In this context, the diffusion of gas out of a nanobubble is inhibited by the supersaturation of dissolved gas due to the dissolution of surrounding bubbles. This was stressed by the molecular dynamic simulations of Weijs *et al.*⁹ They showed that for sufficient gas supersaturation, nanobubbles nucleate in bulk and the clusters of bulk nanobubbles are stabilized by the presence of surrounding nanobubbles, as they shield the diffusive outflux of gas molecules.⁹ When the bubble concentration is decreased, i.e., increasing the bubble–bubble distance, nanobubbles are no longer stable. The simulations demonstrate a critical relationship between the amount of gas present and the ratio of bubble radius R to interbubble separation l , R/l being constant for a constant amount of gas in the system (see Figure 1.6). This means that for a given amount of gas, bubbles can shrink (grow) if the bubble–bubble separation shrinks (grows). Note, however, that the simulation is done in a closed system, whereas in experiment the systems are open or at least partially open.

To sum up the above, so far the mechanism that stabilizes bulk nanobubbles is not known and it appears to lie beyond classical surface

thermodynamics. From this author's point of view, no individual theory can explain the stability of bulk nanobubbles, and it is more likely due to a combination of a number of mechanisms, depending on the specific conditions of the system. Further studies are needed. However, this is *not* the focus of this thesis, our goal being instead the study of interactions of bulk nanobubbles with other types of matter.

Figure 1.6: Nondimensional equilibrium radii $R_{eq}l^{-1}$ of bulk nanobubbles as a function of gas supersaturation, where l is the bubble–bubble distance, N_g is the number of the gas atoms and N_g^* is the critical amount of gas atoms required to prevent full dissolution of a nanobubble. Reprinted and adapted with permission from J.H. Weijs *et al.*; *ChemPhysChem*, **2012**, 13, 2197–2204. Copyright © 2012 John Wiley and Sons.

1.1.4 Concerns about contamination

There is still skepticism about nanobubbles.^{88, 89} Perhaps the most controversial is “Are they really made of gas?” Apart from the hypothesis that nanobubbles are coated with a contamination skin, another suggestion in the literature is that they are purely polymeric contaminants.^{12, 75, 78, 89} Berkelaar *et al.* exposed surface nanobubble-like objects to a degassed environment and found that the nanobubbles remained stable. After thorough

investigation, they figured out that the nanobubble-like objects were induced by the use of disposable needles in which PDMS contaminated the water.⁷⁵ Regarding the bubbles generated via water electrolysis, one might argue that they are arising from the electrode degradation. We address this concern in Chapter 3. In short, contamination needs to be taken seriously and clean techniques are required. As a starting point, the use of disposable syringes should be avoided.

1.2 Experimental studies of applications of bulk nanobubbles

Nanobubbles are especially interesting as they pose a number of challenges to our understanding of bubble features. On the other hand, they may prove useful. The presence of bulk nanobubbles has implications on varieties of interfacial features and behavior of water, thus it is relevant for all applications where these features matter. This includes for example ultrasound contrast agent,^{24, 90, 91} froth flotation,^{92–97} wastewater treatment,^{49, 50, 98, 99} cleaning,³⁹ drug delivery,^{90, 100} disease diagnosis¹⁰¹ etc. Here we focus on three particularly promising ones.

1.2.1 Froth flotation and wastewater treatment

Froth flotation is an effective separation method based on the differences in surface hydrophobicity of different particles and has been widely applied in industrial processes, such as mining and wastewater treatment.^{49, 50, 92, 97, 102–112} Gases are inherently hydrophobic, thus the recovery in froth flotation begins with the collision and adhesion of hydrophobic particles to the gas bulk bubbles followed by transportation of the hydrophobic nanoparticle–bubble complex from the collection zone to the froth zone, enrichment of the froth, and finally removal from above by overflow, while

hydrophilic particles remain and are discharged as tailings.¹¹³ Fan *et al.* found that the presence of nanobubbles in flotation slurry expands the particle size range, increases the particle hydrophobicity and as a result, improves the probability of coal and phosphate flotation recovery.¹¹⁴ In the context of flotation recovery, it is most efficient for particles within a certain size range, nominally from 50 μm to 600 μm for coal and from 10 μm to 100 μm for minerals.^{115, 116} This requires the capability of initiating the growth of nanobubbles into microscopic or even macroscopic bubbles. Schubert and Gong reported that nanobubbles can form on hydrophobic particle surfaces, exist there and even grow into microbubbles, facilitating the particle precipitation by buoyancy.^{117, 118} In a series work of Fan *et al.*, they concluded that the stability of microbubble suspensions can be enhanced by adding nanobubbles with a more remarkable effect under lower frother concentration. Moreover, the presence of nanobubbles helps reduce the bubble rising velocity, which creates more favorable conditions for froth flotation of hard-to-float particles that require a relatively long slide time.^{93–96}

Bulk nanobubbles are also applied in wastewater treatment.^{49, 50, 92, 112, 119, 120} Uchida *et al.* applied bulk nanobubbles in the wastewater of a sewage plant and demonstrated that bulk nanobubbles adsorb impurities in solutions effectively and concentrate them on their surface, which leads to separating impurities from solutions. Their TEM images suggested that the sweep area of nanobubbles in bulk is limited and it is strongly dependent on the number density and lifetime of bulk nanobubbles.⁵⁰ In another study, Xiao *et al.* studied the impact of nanobubbles on phosphine recovery in artificial wastewater. They revealed that the role which bulk nanobubbles plays in the precipitation and flotation is different. Nanobubbles inhibit the precipitation of styryl phosphoric acid (SPA)–Pb particles with small size ($\sim 1\text{ nm}$), whereas when SPA–Pb particles are large ($\sim 2\text{ }\mu\text{m}$), nanobubbles promote the flotation of precipitated SPA–Pb particles. Furthermore, adding nanobubbles into precipitated SPA–Pb particles could enhance the efficiency of flotation recovery up to 90 %.⁴⁹

1.2.2 Cleaning

Incorporating gas bubbles in the water used for cleaning has been reported for diverse substrates such as silicon wafers, mica, nickel-plated surfaces, highly oriented pyrolytic graphite (HOPG), gold surfaces, stainless steel coupons and polypropylene pipe.^{39, 43, 98, 105, 121–125} Burfoot *et al.* first showed that adding bubbles into cleaning water during ultrasonic cleaning improves the removal of biofilm, carbohydrate, fat and protein deposits from steel surfaces. They suggested that the cleaning is due to the action of the bubbles rather than the cavitation events associated with ultrasonic cleaning.^{105, 126} Similarly, Takenouchi *et al.* demonstrated the rinse effect of hydrogen nanobubbles in alkaline electrolyzed water (AEW). They showed that AEW cleans and removes sulfate ions efficiently from the nickel-plated surface due to the ion exchange reaction between hydroxide ions located at the nanobubble interface and the sulfate ions remained on the nickel surface.⁴³ A recent study by Zhu *et al.* investigated the cleaning efficacy of bulk nanobubbles in the removal of model contaminants bovine serum albumin and lysozyme from silicon wafers with opposite wettability (hydrophilic and hydrophobic). They found that bulk nanobubbles can prevent the fouling of both surfaces and remove the contamination from hydrophilic surfaces but not from hydrophobic surfaces. The latter was attributed to the strong adhesion between the protein molecules and the hydrophobic surface.³⁹

1.2.3 Ultrasound contrast agents

Another possible use is as ultrasound contrast agents.^{24, 91, 127} Ultrasound contrast agents have been developed to artificially change the reflection coefficient between the two tissues, thus enhancing the backscattered signal and improving the image contrast.¹²⁸ In general, ultrasound contrast agents are micron-sized stabilized gas bubbles, allowing for imaging in both the venous and arterial vessels.^{129–132} To penetrate beyond the vasculature, such

as tumors and inflammation, the contrast agent needs to be over an order of magnitude smaller, i.e. in the nanometer range.^{24, 90, 91, 127} Preliminary studies conducted by Xing *et al.* and Wheatley *et al.* using nanobubbles as contrast agent exhibited excellent power Doppler enhancement *in vivo* renal imaging in a rabbit.^{91, 127} Rapoport *et al.* developed an echogenic drug delivery system combining polymeric micelles, nanobubbles, and microbubbles. They found that small nanobubbles are capable of extravasating into the tumor, as tumors are characterized by a defective vasculature that have enhanced permeability to nanoparticles up to 750 nm. On accumulation in the tumor tissue, small nanobubbles induced by ultrasound or hyperthermia coalesce into larger highly echogenic microbubbles, resulting in a strong ultrasound contrast in tumor ultrasonography. In their study, bulk nanobubbles also serve as drug carriers in addition to acting as precursors of strong ultrasound contrast agents.⁹⁰

The variety of applications employing bulk nanobubbles shows that they may indeed have practical implications. Yet, the research in this aspect is not systematic. In this thesis, we attempt to fill in this gap by investigating the interactions of bulk nanobubbles with different systems.

1.3 Aims and outline of this thesis

The work reported in this thesis systematically investigates the interactions of bulk nanobubbles with different matter, namely, negatively charged Au nanoparticles, amidine terminated polystyrene beads, liposomes and viral capsid proteins. We experimentally explore how the bulk nanobubbles interact with and essentially influence the charge state of nanoparticles. We further investigate the ability of bulk nanobubbles to drive the self-assembly of viral capsid proteins.

The thesis is organized in 5 chapters:

Chapter 1 gave a general literature review on the subject of bulk nanobubbles, including generation and characterization methods, stability mechanisms and experimental studies of applications.

Chapter 2 investigates the interaction between negatively charged gold nanoparticles and bulk nanobubbles, demonstrating that the interaction is through gas nucleation on the nanoparticles and that it is nanoparticle size dependent.

Chapter 3 reports the ability of gas-saturated bubble solution to influence the charge state of positive nanoparticles added to the solution. Positively charged nanoparticles undergo reentrant condensation upon mixing with bulk nanobubble solutions of different concentrations.

Chapter 4 studies the interaction of bulk nanobubbles with an organic system, namely liposomes. The same reentrant condensation behavior of cationic liposome solutions is presented and fluorescence microscopy is applied to visualize the interaction process.

Chapter 5 focus on the self-assembly of viral capsid proteins induced by bulk nanobubbles using three distinct techniques: dynamic light scattering (DLS), atomic force microscopy (AFM) and transmission electron microscopy (TEM).

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NANOBUBBLE–NANOPARTICLE INTERACTIONS IN BULK SOLUTIONS

Nanobubbles form stable colloids in supersaturated solutions. Here we demonstrate the ability of these solutions to interact with Au nanoparticle suspensions. The principal goal was to demonstrate particle modification, similar to froth flotation, and we do indeed see bubble–particle interactions. However, unlike in froth flotation, where bubble–particle interactions are driven mainly through collisions, for bulk nanobubble solutions we find that the primary interaction is through nucleation of nanobubbles on the particles.

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2.1 Introduction

Nanobubbles are thought to be nanoscopic gas bubbles that occur both on interfaces and in bulk solutions, known as “surface nanobubbles” and “bulk nanobubbles,” respectively.^{1–5} They have experienced a dramatic increase in interest in recent years, driven primarily through their enhanced longevity, with measured lifetimes (typically hours or days) many orders of magnitude longer than a simple diffusive time scale would predict (microseconds).⁶ This has had consequences both fundamentally and in terms of applications. On the one hand, what is causing the greatly enhanced lifetime? Several competing theories have been proposed for nanobubble stability, including contact line pinning (specifically for nanobubbles on surfaces), contaminant ‘armor,’ and diffusive shielding, all of which are thought either to prevent or slow down dissolution.^{7–11} On the other hand, regardless of the stabilizing mechanism, to what extent can we exploit nanobubbles in applications? Already evidence exists that nanobubbles can be used to reduce protein adsorption,¹² that they may be used for nanotemplating,¹³ and that they may interact with soiled surfaces to act as cleaning agents. In terms of applications, to date they are predominantly associated with surface nanobubbles; bulk nanobubbles have been much less investigated.

An obvious question is thus how far can the range of applications of bulk nanobubbles be developed. One clear possible application is in nanoparticle attachment. This can most easily be visualized as nanoscopic froth flotation.¹⁴ In this respect, it was recently demonstrated that nanobubbles could assist in nanoparticle–substrate adsorption¹⁵ and nanoparticle–macrobubble attachment,^{16–19} possibly assisted by capillary bridging.²⁰ While these recent studies point directly toward the use of nanobubbles in applications, it is unclear whether the bubbles in these articles are “nanobubbles” in the present sense. In this respect, yes they are indeed nanoscopic bubbles (albeit with diameters much greater than those in the “nanobubble” literature) but they

do not exhibit the superstability shown by the nanobubbles in all of the other studies mentioned above.²¹ In fact the authors of ref. 16¹⁶ present data showing their nanobubbles growing above micron dimension within ~ 1 min, i.e., they appear to be truly distinct from the rest of the nanobubble literature.

Our goal in the present work is to answer the following question: Can *superstable* nanobubbles interact with nanoparticles, akin to froth flotation? We can help ourselves already by removing the requirement for the particles indeed to “float” to the top of a container for recovery, i.e., the bubbles need not be a buoyancy aid, and replacing it with the ability of the bubbles to simply interact with the nanoparticles. We imagine that such interaction could vary important parameters of the nanoparticle/nanobubble agglomerates (zeta potential, size, diffusion coefficient) for other separation technologies to be implemented for harvest.

Here we demonstrate the ability of bulk nanobubbles to interact with gold nanoparticles. The nanobubbles are generated through high-powered electrolysis of water, which leads to supersaturated oxygen and hydrogen gas that can then nucleate bubbles. We discover that, unlike in froth flotation, where the bubble–particle interaction is principally through collision, bulk nanobubble solutions interact through nucleation on the nanoparticles. This means that bulk nanobubbles are thus able to modify colloidal suspensions.

2.2 Experimental Methods

2.2.1 Nanobubble generation and characterization

The nanobubble solutions for the data presented here were created from Milli-Q water (Millipore, USA) with 10 mM NaCl dissolved. We also experimented with NaCl concentrations from 10 μ M to 100 mM, as well as with phosphate buffer solution, but the results agreed with the original NaCl 10 mM solution within error, so we proceeded with those.

Generation of the nanobubble solution was achieved via high power water electrolysis using a commercial electrochemical flow cell. Briefly, this consists of several macroscopic electrode plates made of Pt on etched Ti, which are separated from each other by several mm. The geometry is a flow-through cell, i.e., water is delivered at one end of the generator, flows between the plates while being electrolyzed, and exits the cell downstream. During electrolysis the water is broken into oxygen (see Figure 2.1(a)) and hydrogen gas, both of which are allowed to dissolve into the water stream (note that the anolyte and catholyte are not separated from each other, i.e., the exiting water is a single stream and contains a mixture of both O₂ and H₂).

We note that the freshly generated liquid contained several visible macroscopic bubbles that floated to the top of the container within a few seconds. The data in this article refer to the nanoscopic bubbles that remain in the bulk liquid once these other bubbles have exited, i.e., to the bubbles sufficiently small for diffusion to effectively compete with buoyancy over the experimental time scale.

Immediately upon generation, the outlet water stream was supersaturated with O₂ to $\sim$ 10 mg/L as compared to 8.9 mg/L at equilibrium (measured by an oximeter, PreSens).

Characterization of the nanobubble solution was carried out using dynamic light scattering for sizing (DLS; Zetasizer Nano ZS, Malvern), nanoparticle tracking analysis for bubble lifetime and concentration (NTA NS500, Nanosight), and electrophoresis for zeta potential (Zetasizer Nano ZS, Malvern), all with thermal control at 25 °C. Experimental parameters for each of the techniques were as follows:

- DLS: Measurement time of 10 s, repeated continuously for 8 h. Sample holder: square polystyrene cuvettes (DTS0012, Malvern). Measurement volume (“field of view”): 1.4 nL. Liquid held stationary.
- NTA: Measurement time of 60 s, repeated continuously for 180 s. Measurement volume (“field of view”): 80 pL. Both liquid flowed steadily at 600 L/min with a syringe pump (Harvard Apparatus), and liquid held stationary (note that background flow was subtracted from particle tracks when analyzing diffusion coefficients and particle sizes).
- Electrophoresis: Measurement time of 10 s, repeated continuously for 100 s time. Sample holder: Folded capillary cell (DTS1061, Malvern).

2.2.2 Investigation of nanobubble–gold nanoparticle interactions

To investigate nanoparticle–nanobubble interactions we used Au nanoparticles with nominal diameters 10, 20, 30, 40, 60, 80, and 100 nm, supported in PBS (Sigma-Aldrich). These nanoparticles have central solid cores (to which the listed diameters correspond), which are surrounded by rough asperities. In total, the hydrodynamic diameters, i.e., the “equivalent” sphere sizes that they dynamically respond with (solid core + roughness + hydration layers), are typically 10–20 nm larger than the core sizes (see Table 2.1).

Table 2.1: Size information for the pure nanoparticle solutions. The ranges of core and hydrodynamic diameters given by the manufacturer, as well as the peak size measured here using DLS. The measured values given in brackets represent the full width at half maximum of the distributions.

solution	core size (nm)	hydrodynamic size (nm)	measured size (nm)
10 nm Au nanoparticles	8–12	11–25	16 (8-26)
20 nm Au nanoparticles	18–22	21–32	36 (19-67)
30 nm Au nanoparticles	28–32	31–43	44 (23-62)
40 nm Au nanoparticles	37–43	41–53	53 (31-90)
60 nm Au nanoparticles	57–63	61–73	78 (45-133)
80 nm Au nanoparticles	77–83	81–93	94 (58-151)
100 nm Au nanoparticles	98–115	100–125	108 (73-160)

The nanoparticle solutions were ultrasonicated for 1 min prior to mixing them with the nanobubble solution. Preparation of the nanoparticle–nanobubble mixtures was carried out by gently mixing the two pure solutions at a specific particle-to-bubble number ratio. Bearing in mind that the optical characterization techniques used here partially prioritize data based on intensity of light reflection, the different reflectivities of Au nanoparticles and gaseous nanobubbles may lead to one population being hidden in the noise of the other population. With this in mind, we tested particle-to-bubble ratios of 100:1, 10:1, 1:1, 1:10, and 1:100. It was clear that one population of objects became hidden in the noise of the other at the extreme ratios, but the ratio of 1:1 gave two distinct population peaks, so we present the 1:1 ratio data here for all Au nanoparticle sizes.

Once the nanoparticle-nanobubble mixtures had been prepared, we proceeded to interrogate the new solutions with either DLS or NTA. For DLS we measured the system for several hours, but the system stabilized typically within the first data point, i.e., a few seconds. This stabilization time was on top of the ~ 20 – 30 s required to transfer the liquid from the generation unit to the measurement equipment.

All liquids were handled using 10 mL glass syringes (Fortuna Optima) and we flowed the solution through 450 nm PVDF microfilters (Fisher Brand) prior to the nanobubbles being generated.

2.3 Results and Discussion

2.3.1 Characterization of the nanobubble solutions

We begin by characterizing the pure nanobubble solution. The size probability distribution function from DLS is plotted in Figure 2.1. We found nanobubbles with a single, broad distribution, with peak diameter and full width at half maximum of 223 nm and 94–529 nm, respectively, which is consistent with other nanobubble studies in the literature.^{22, 23} Furthermore, the solution as a whole exhibited long term stability, as shown by continually repeating the DLS measurement over a 4 h period (see Figure 2.1(c)).

The NTA size data (not shown) are qualitatively similar to the DLS data, although the peak diameter and standard deviation are smaller. We ascribe the quantitative difference between the DLS and NTA data as coming from the implementation of the two techniques: In NTA we measure the motion of a relatively small number of individual objects (see Figure 2.2(a)), then calculate diffusion coefficients and sizes for each individual object. In DLS we measure the collective diffusion of a larger number of objects, then calculate their average size. NTA thus has the advantage of being able to accurately

Figure 2.1: (a) Nanobubbles are formed through high power electrolysis which supersaturates the water with dissolved gas. (b) DLS size results for the pure nanobubble solution. Nanobubbles have a peak diameter of 223 nm with full width at half-maximum of 94–529 nm. (c) Many DLS size distributions as a function of time demonstrate the solution’s long time stability (color scale runs from minimum probability (dark) to maximum probability (light)).

measure polydisperse populations but has the disadvantage of only sampling a small number of the population; DLS benefits from sampling a much larger population but loses out in terms of assuming a monodisperse distribution in the analysis. Furthermore, DLS measures the behavior of the sample in the bulk in stationary conditions, whereas NTA measures the behavior in a much smaller cell with background flow. Satisfied that both techniques agree with each other within the bounds of this caveat, we proceed by using DLS for sizing measurements and restrict NTA to concentration measurements (NTA has the advantage that by measuring the exact number of particles

within a given volume, the number density can easily be found). For the solution generated by our flow cell we find a nanobubble number density of $\sim 10^7\text{--}10^8/\text{mL}$, corresponding to an average bubble–bubble spacing of $L \sim 20\text{--}50\text{ }\mu\text{m}$.

Figure 2.2: (a) Thirty one individual nanobubble tracks from NTA. Pixel dimensions correspond to $\sim 100\text{ }\mu\text{m}$ in the x-direction and $\sim 80\text{ }\mu\text{m}$ in the y-direction. Data is recorded at video rate (25 fps) and we are only presenting data for tracks lasting longer than 1 s. The nanobubbles individually exist much longer than the diffusive time scale, indicating that they are stable to dissolution. (b) According to DLVO theory the nanobubbles are colloiddally stable since the potential barrier W is much greater than thermal energy $k_B T$. The nanobubbles can never approach each other closer than the typical electrostatic (Debye) screening length, λ_D .

The long term stability exhibited by the DLS data of Figure 2.1(c) could either correspond to the nanobubbles being individually stable, or to the *global* number of bubbles present in solution to be steady, but with this maintained through local populations undergoing constant dissolution and nucleation. Such a “flickering” effect of constant dissolution/nucleation would occur within a typical diffusive time scale of $\tau_{\text{dis}} \sim R^2/D \sim 10\text{ }\mu\text{s}$, where we have used a typical bubble radius $R \sim 100\text{ nm}$ and gas diffusion coefficient $D \sim 10^{-9}\text{ m}^2/\text{s}$. In Figure 2.2(a) we show how individual nanobubble tracks recorded by NTA exist for tens of seconds, i.e., the solution is stable to the individual bubbles dissolving. In fact we did not measure bubble dissolution at all-particle tracks in Figure 2.2(a) that end abruptly are due to nanobubbles

moving out of the field of view of the NTA measurement window.

From the above, individual nanobubbles are stable to dissolution (from the NTA data) and the bulk nanobubble population size distribution is stable (from the DLS data). We now go one step further and ask whether or not the bubbles collide or merge with each other (perhaps separating later or partially dissolving leading to new nucleation to maintain an overall equilibrium number), i.e., are they colloidally stable? The expected bubble–bubble collision time is $\tau \sim L^2/D \sim 1000$ s, where D is the bubble diffusion coefficient found from the Stokes–Einstein relation, $D = k_B T / 4\pi\eta R \sim 3 \times 10^{-12}$ m²/s, with $k_B T$ the thermal energy and η the viscosity of water (1 mPas). The fact that the bubble size distribution does not change over this time scale in Figure 2.1(c) is highly indicative that the nanobubbles are colloidally stable. To prove colloidal stability we estimate the energy barrier preventing bubble–bubble collision from the DLVO theory. The attractive van der Waals interaction potential of $U_{\text{vdw}} = -AR/12d$ competes against the repulsive electrostatic double layer interaction potential of $U_{\text{edl}} = RZe^{-d/\lambda_D}/2$, where A is the Hamaker constant, D is the Debye length (~ 3 nm), and $Z \approx 10^{-10} \tanh^2(ze\psi_o/k_B T)$ is the amplitude of the electrostatic repulsion, with z the electrolyte valency of 1 for NaCl, e is the unit charge of 1.6×10^{-19} C, and ψ_o is the potential of a bubble surface. Using the measured zeta-potential of the nanobubbles, $\zeta \approx -20 \pm 3$ mV, as a lower bound for ψ_o and $A = 3k_B T$ for indicative purpose,²⁴ we predict the energy barrier preventing bubble–bubble collision to be $\sim 25 k_B T$, confirming the fact they are indeed colloidally stable (see Figure 2.2(b)).

To summarize the above, the nanobubbles in our solution are stable against dissolution (both as an entire population and individually) and against bubble–bubble collision/merger.

2.3.2 Nanoparticle-nanobubble interactions

We now address whether or not the nanobubbles in suspension interact with the Au nanoparticles in suspension. We present DLS data for the mixture of 20 nm nanoparticles and nanobubbles in Figure 2.3(a). Two distinct size distributions exist corresponding to the two original species, i.e., the nanobubbles and nanoparticles do not appear to interact. This is perhaps of no surprise: Nanobubbles and nanoparticles are independently stable, and we expect the same to be true for their mixtures. In fact to further highlight their stability, in Figure 2.3(b) we show a time series of DLS data over 8 h; the two entities do not interact with each other for an extended period, orders of magnitude longer than the diffusive collision time. (Note that the difference in peak heights for the nanoparticle and nanobubble distributions in Figure 2.3(a) arise due to slight mismatches in number density between the two species, as described in the Experimental Method).

The principle aim of this paper is to demonstrate that nanobubbles *can* interact with nanoparticles, so how can we reconcile ourselves with our initial observation? It turns out that *larger* nanoparticles behave differently than the 20 nm particles. As an example we present the size data of the solution of 60 nm Au particles with nanobubbles in Figure 2.3(c). The principle finding is that, upon mixing these larger particles with our nanobubble solution, we no longer see either pure Au particles or the original nanobubbles (both peaks are no longer present in the DLS data). However, a new peak does appear at an intermediate size and it remains stable for several hours with no obvious peak shift or broadening during the entire 8 h measurement window.

We interpret this new peak as a combination/agglomeration of nanobubble and nanoparticle. Our reasoning is as follows: The Au nanoparticles cannot easily change their size through aggregation (they are colloiddally stable with a very high energy barrier). The fact that we no longer see the nanoparticle peak then implies that they must have attached to the nanobubbles. We anticipate

Figure 2.3: DLS size distributions and long-term dynamics of 20 and 60 nm Au nanoparticles in nanobubble solution. The 20 nm Au nanoparticles do not interact with the nanobubbles, as shown by the two separate size distributions in (a) corresponding to distinct nanoparticle and nanobubble populations. On the other hand the 60 nm Au nanoparticles interact with the nanobubbles to form new entities with intermediate sizes (c). In both cases the solutions are stable over at least 8 h (b and d for 20 and 60 nm nanoparticles, respectively).

that this would be a nanobubble decorated with a few (one?) nanoparticles on its gas/liquid interface rather than, e.g., large agglomerations of nanoparticles connected through bridging gas layers (see below for our reasoning). Why is this nanoparticle–nanobubble agglomeration smaller than the sum of its two parts? Unlike nanoparticles, nanobubbles are able to vary their size through diffusive growth or decay, depending on local conditions. In the present case, it appears that they have partially decayed.

We now face a dilemma in our understanding. We intuitively imagine that

the 60 nm nanobubbles may diffusively collide with the nanoparticles, similar to froth flotation. However, the 20 nm system is colloidally stable, i.e., the energy barrier preventing aggregation is too high for the 20 nm nanoparticles plus nanobubbles as compared to thermal energy to allow collision. The problem is that both the electrostatic repulsion and van der Waals attraction of DLVO theory scale linearly with particle size, so increasing the particle size (from 20 to 60 nm in this example) leads to an increase in energy barrier preventing collision, i.e., if the 20 nm system is colloidally stable, then all of the larger nanoparticle systems must be more stable to collisions, i.e., the nanoparticle–nanobubble agglomerations cannot arise through collisions.

So how to proceed? We first address why the nanoparticle–nanobubble agglomerations are smaller than the sum of their two parts. In ref. 25 we demonstrated a critical relationship between the amount of gas available (both in the bubbles and dissolved) and the ratio of bubble to interbubble separation, i.e., R/L is constant for a constant amount of gas in the system.²⁵ This means that, for a given amount of gas per unit volume, bubbles can shrink (grow) in size as long as their separation also shrinks (grows) by the same relative amount. How does the interbubble separation shrink? This is only possible if more bubbles nucleate. Hence, in our experiments described here, the nanobubbles can shrink from before mixing to after mixing as long as additional bubbles nucleate during the mixing process. New nucleation explains the observation.

Now that nanoparticle–nanobubble interactions are understood for the 60 nm particles we proceed to look as a function of particle size. The DLS size data are presented in Figure 2.4. It is clear that all of the nanoparticles with diameter 40 nm or above behave similarly to the 60 nm particle systems, i.e., the nanoparticles and nanobubbles combine to form a new entity larger than the original particles but smaller than the original nanobubbles. However, all systems with nanoparticles of diameter 30 nm or below behave similarly to the 20 nm nanoparticle systems.

Figure 2.4: Size distributions of nanoparticle–nanobubble mixtures as a function of nanoparticle size. Two distinct populations exist in solutions with smaller nanoparticles, whereas a single population exists in solutions with larger nanoparticles. The crossover occurs for nanoparticles between 30 and 40 nm.

The final piece of the puzzle is to address why the smaller nanoparticles do *not* allow nucleation of bubbles. It is well-known from nucleation theory that a critical minimum size is required for nucleation to take place. The creation of new volume (here a gas bubble) increases the overall energy of the system. However, the interfacial area around the bubble is energetically costly. Nucleation is expected when the energy gained through added volume, V , outweighs the cost from added area, A . The cost in free energy for nucleation is then given by $\Delta G_{\text{homo}} = V\Delta g + A\gamma_{lg}$, with Δg the difference in free energy per unit volume of the gaseous phase versus the gas-dissolved-in-water

phase, and γ_{lg} is the surface tension of the water/gas interface (~ 73 mN/m at room temperature). In the case of heterogeneous nucleation, i.e., on a solid surface, the surface catalyzes the nucleation by providing an interface with a lower surface energy than that of water/gas (heterogeneous nucleation also benefits hugely from the addition of rough asperities to nucleate on); the loss in volume from the nucleated bubble not being fully spherical is more than accounted for by the reduction in energy cost from nucleating on an energetically cheaper surface. The overall cost in free energy for nucleation in this case is $\Delta G_{\text{hetero}} = \Delta G_{\text{homo}} f(\theta)$, with $f(\theta) = (2 - 3 \cos \theta + \cos^3 \theta)/4$,²⁶ where θ is the contact angle. This additional factor accounts for the geometrical differences listed above.

In the present case, heterogeneous nucleation on a planar surface (see Figure 2.5(a)) does not explain the differences observed as a function of nanoparticle size. To explain our observation, we note that if the nucleation surface is not planar, then the shape of the surface changes the energy barrier from that for the planar case. If we assume that our nanoparticles are spherical to first order, then nucleation is less favorable on the nanoparticles than on a planar surface. This is because the nanoparticle protrudes into the bubble's volume as compared to the planar case (see Figure 2.5(b)). Hence, the bubble's volume is smaller than before, i.e., volume creation gains less energy as compared to the planar case. Also, the solid/gas interfacial area is slightly larger, i.e., area creation costs more energy as compared to the planar case. The nucleation barrier on a spherical nanoparticle thus lies between the planar heterogeneous case and the homogeneous case. In this case ΔG does not have a straightforward expression, but we plot the result in Figure 2.5(c) with $\Delta g = -2 \times 10^7$ J/m³ and $\theta = 70^\circ$ for indicative purposes.^{26–29} While precise surface energies are unknown for us to make an accurate estimate of the exact critical seed size, the trend is in the right direction and qualitatively explains our observation.

Figure 2.5: (a) Heterogeneous nucleation on a planar surface is energetically more favorable than homogeneous nucleation. Although less volume is created, the solid/gas interface is cheaper to create than liquid/gas. (b) Heterogeneous nucleation on a spherical nanoparticle is energetically less favorable than on a planar surface. The nanoparticle protrudes into the bubble volume (here, the region above the dashed line), meaning less volume is created. Also, the surface area of the footprint of the bubble on the solid is larger than for the planar case. (c) Energy barrier preventing nucleation for homogeneous nucleation (red, top), heterogeneous nucleation on a planar surface (black, bottom), and heterogeneous nucleation on spherical nanoparticles (blue) as a function of nanoparticle diameter. Nucleation on smaller nanoparticles costs more energy than on larger nanoparticles.

Our last measurements are of the zeta-potential of the nanoparticle–nanobubble solutions. The data are presented in Figure 2.6. For each nanoparticle size, the overall result of mixing the nanoparticles with the nanobubble solution is a decrease in absolute value of zeta-potential. This is to be expected since, after nucleation, we expect nanobubbles to partially screen out the nanoparticles’ surface charges. This confirms our initial prediction that nanobubbles can modify material properties of nanoparticles, which maybe utilized downstream for nanoparticle segregation.

Figure 2.6: Zeta-potential measurements for pure Au nanoparticle solutions (red, dashed line) and nanoparticle/nanobubble solutions (blue, solid line). The particle/bubble solutions have smaller magnitude zeta-potentials than the pure particle solutions, verifying that the nanobubbles are able to modify the particle properties.

2.4 Conclusion

We have shown that bulk nanobubble solutions interact with nanoparticles. Unlike froth flotation, where interaction is principally through bubble–particle collision, for the case of nanobubbles the interaction is through nucleation on the nanoparticles. This means that pre-existing nanobubbles do not play a major role in nanoparticle–nanobubble interactions.

Our finding of nucleation versus collision is supported by the fact the smallest nanoparticles do not interact with nanobubbles. If collisions were the driving force we would expect the smallest nanoparticle to interact the most due to both the reduced DLVO energy barrier preventing collision and the increased diffusive speed allowing larger liquid volumes to be sampled in a given time.

The ability of nanobubbles to interact with nanoparticles opens the door for enhanced scavenging through different separation technologies, as well

as surface modification. Another major application that we foresee is in industrial cleaning. Here, mechanical brushes lift dirt from surfaces into suspension in water. Nanobubbles can then nucleate on the dirt particles and prevent them from redistribution on the surface. Nanobubbles thus provide a potential cleaning mechanism that can be free from detergents and other chemical agents.

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REENTRANT CONDENSATION OF AMIDINE LATEX NANOPARTICLES DRIVEN BY BULK NANOBUBBLES

High-power water electrolysis converts water into an aqueous solution supersaturated with oxygen and hydrogen. Here we demonstrate the ability of this gas-saturated solution to influence the charge state of positive nanoparticles, resulting in a decrease or even reversal of their electrophoretic mobility. The nanoparticles further undergo reentrant condensation close to their point of zero charge, as directly observed via an increase in apparent size and macroscopic aggregation. We attribute this behavior to gas bubbles interacting with the nanoparticles via nucleation on their surface.¹

3.1 Introduction

3 Gas supersaturated solutions have attracted a lot of recent attention with the observation of stable colloids in saturated solutions following water electrolysis.^{2–5} The existence of these nano-sized gas bubbles was first proposed by Parker *et al.* to explain the long-range (~ 100 nm) attractive force between two hydrophobic surfaces immersed in water.⁶ Since then, evidence of both surface nanobubbles (nanoscopic gaseous bodies formed at solid/liquid interface)^{2, 7–9} and bulk nanobubbles (gas filled nanoscopic spherical bodies in bulk liquids)^{3, 7, 10–13} was reported extensively utilizing various techniques that include atomic force microscopy (AFM),^{14–16} infrared spectroscopy,¹⁷ transmission electron microscopy (TEM),¹⁸ Cryo-EM,¹⁹ and light scattering.^{1, 20} The general observation is that gas filled spherical particles are formed with a diameter ranging from tens to hundreds of nanometers that can survive for hours or even days in aqueous solution.^{3, 13, 21}

Their long-term stability is a paradoxical property of bulk nanobubbles. Theoretically, bubbles with radii of curvature lower than $1\ \mu\text{m}$ would disappear within microseconds attributed to the high Laplace internal pressure,^{22, 23} whereas measurements indicate much longer lifetimes.^{3, 7, 10} A number of explanations have been proposed for this long-term stability including supersaturation, diffusive shielding, contaminant protective cover, and absorption of hydrophobic particles or ions at the gas-water interface.^{3, 12, 24–26} These mechanisms assume cooperative effects within bubble clusters and/or the reduction of their surface tension at the air/liquid interface. There is however currently no generally accepted mechanism for the long term stability of bulk nanobubbles.

Recently we have shown the interactions of bulk nanobubbles with negatively charged gold nanoparticles.¹ Our experiments employ high-power water electrolysis, which generates a large amount of supersaturated oxygen and hydrogen gas that can participate in bubble nucleation. This results in

stable, nanobubble-like bodies with properties consistent with other reported findings^{2, 3} and which we therefore refer to as bulk nanobubbles here. We emphasize that our primary focus here is not on the stability mechanism, however, but rather on studying the interactions between gas supersaturated solutions and charged nanoparticles. Unlike froth flotation, where bubbles and particles interact mainly via attachment, we previously demonstrated that bulk nanobubble solutions interact with gold nanoparticles through nucleation on the gold surface, forming a new single population of particles with a diameter intermediate between those of the original nanoparticles and nanobubbles. This effect is highly dependent on the size of the gold nanoparticles, with particles sized 30 nm or less exhibiting no effect.

If our hypothesis is true that nucleation of bubbles on particles is the main mode of interaction, then interactions with positively charged particles can be expected to be significantly stronger. This is because the surface of nanobubbles, like their larger counterparts, has been shown to exhibit a negative charge.²⁷ Any change in net surface charge can be straightforwardly monitored through measuring the electrophoretic mobility of nanoparticle–nanobubble mixture solutions to extract their zeta potential.

In this Chapter, we comparatively study the interactions of gas supersaturated solutions with both positive and negative nanoparticles employing amidine-modified polystyrene (PS) latex nanoparticles as positive nanoparticle model. We demonstrate the ability of bulk nanobubble solutions to tune the electrophoretic properties of positive nanoparticles by tuning the particle–nanobubble ratio. This effect can be so pronounced as to cause charge inversion. In a finite range of concentrations near the point of zero charge, the particles lose colloidal stability and aggregate. Control experiments in the absence of gas supersaturation do not exhibit this behavior. We believe that understanding these interactions between charged colloidal particles and charged nanobubbles can help elucidate both the phase behavior of charge stabilized colloids and proposed applications of nanobubbles such as the

mechanism behind nano cleaning.²⁸

3.2 Experimental Methods

3.2.1 Nanobubble generation

Sodium chloride solutions of concentration 10 mM were prepared with milli-Q water (Millipore). The nanobubble solutions were then created by high power water electrolysis in an electrochemical flow cell. Briefly, this employs five macroscopic electrode plates made of platinum on etched titanium with a plate spacing of 2.79 mm. The salt solution was delivered at one end of the flow cell, flowed through the plates and being electrolyzed in the meantime, finally exited the flow cell downstream. During electrolysis, the aqueous solution was treated electrochemically in a two-electrode circuit configuration with a cell voltage of 24 V, an average current of 3 A, and a flow rate of 500 mL min⁻¹. The water electrolysis process splits water into oxygen and hydrogen gas, both of which dissolve back into the water stream and cause supersaturation with oxygen and hydrogen. We measured the supersaturation level of oxygen at the outlet water stream to be 110%–130% supersaturated (Fibox 3 trace 3, Fiber optic trace oxygen transmitter, PreSens). The nanobubble solution is stable on the scale of days.

3.2.2 Characterization of nanobubble solutions

Characterization of the nanobubble solution was carried out by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments) for sizing, electrophoresis for zeta potential (Zetasizer Nano ZS, Malvern Instruments) and nanoparticle tracking analysis (NTA, NS500, Nanosight, Malvern Instruments) for concentration, all implemented at 25 °C. Detailed experimental parameters of each technique were as follows:

DLS: The measurements were conducted in accordance with a previous publication.¹ Briefly, five measurements of 60 s duration were performed on each sample. The average and standard deviation of these measurements were used for further analysis. DLS yields particle size in terms of hydrodynamic diameter; our solutions exhibited a single-peak distribution, with peak diameter and full width at half maximum of 223 nm and 94–529 nm, respectively.

NTA: Each measurement comprised an analysis of three movies, each 60 s long and captured at a speed of 25 frames/s. The concentration was directly determined by counting the number of particles tracked in a known volume. For the solution generated by our commercial flow cell, we measured a concentration of $\sim 10^7$ – 10^8 particles/mL.

Electrophoresis: Three measurements were taken for each sample and each measurement took 60 s. The average and standard deviation of 3 measurements were used for further analysis. Sample holder: Folded capillary cell (DTS1061, Malvern Instruments). We measured the pristine nanobubble solution to be negatively charged with a mean zeta potential of -19 ± 3 mV.

3.2.3 Exclusion of platinum electrode degradation

The electrolytic cell that we employed includes platinum-coated planar electrodes which would degrade over time given the harshness of the process. It is therefore important to verify that the platinum nanoparticles liberated from the electrode are not an important source of nanoparticles (incorrectly interpreted as nanobubbles) in our bulk solution. For this purpose, we carried out scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX) elemental analysis.

SEM-EDX: The SEM samples were prepared by drop casting 20 μ L of a freshly generated nanobubble solution onto a 1 cm $\times$ 1 cm wafer

substrate. The samples were then dried overnight under vacuum before being imaged in a field-emission scanning electron microscope (Zeiss MERLIN HR-SEM, Enschede, The Netherlands) and subjected to energy dispersive X-ray spectrometry (Zeiss MERLIN HR-SEM, Enschede, The Netherlands).

Figure 3.1: SEM-EDX analysis of the nanobubble solution contents. Left: SEM image and regions selected for further EDX analysis. Right: two typical EDX spectra.

Figure 3.1 presents both SEM and EDX analysis. Several high contrast regions of the SEM image (on the left) were selected and subjected to EDX analysis. Two typical examples are shown on the right. The SEM-EDX instrument has a detection limit of 0.2% atom fraction. If we regard all the nano bodies inside the solution as metal electrode contaminants, their atom percentage in the system is also close to 0.2%. But rather than being randomly distributed, these atoms would be locally concentrated in the form of easily detectable metal particles. Taking the nano body concentration by NTA into account (Chapter 2), we estimate that every $10\ \mu\text{m} \times 10\ \mu\text{m}$ of the dry salt sample would contain a particle. These would scatter intensely and be readily observable against the relatively inert salt background.

The SEM analysis of the dried solution contents on the left of Figure 3.1 shows no metal inclusions with a diameter $\sim 200\ \text{nm}$, as would be expected

if the nanobubbles were contaminants from the electrolysis process. The EDX analysis on the right side shows that only sodium and chloride were present in the solution (silicon and oxygen signals were also detected from the wafer substrate). These measurements strongly suggest that the suspicion that the electrolysis process in our flow cell is producing a stream of platinum nanoparticles caused by platinum electrode degradation can be excluded.

3.2.4 pH of amidine latex nanoparticle solutions mixed with nanobubbles

Another possible artifact is pH changes induced when mixing nanoparticle and nanobubble solutions. To exclude this possibility, we monitored the ζ -potential of 100 nm stock amidine latex nanoparticle solutions as a function of pH in 10 mM NaCl at 25 °C, as shown in Figure 3.2(a). HCl and NaOH solutions were added as required to adjust pH. The particles bear positive charges originating from amidine groups, but the ζ -potential decreases as pH increases due to the protonation of amidine groups. A charge change in the ζ -potential from positive to negative occurs at high pH with an isoelectric point (pI) in the range of pH 9.0-10.0 (a linear extrapolation gives an estimate for the isoelectric point of pH 9.5.). This shows that the protonated amidine groups on the surface of the particles can be neutralized. Above the pI, the ζ -potential shows a negative sign as pH further increases which could be attributed to the hydrolysis of amidine groups, producing carboxylic groups at the surface. For comparison, the left and right arrows in the figure indicate the pH values of sodium chloride solution before and after it went through the flow cell, respectively.

As a further control, we measured the pH of a series of nanoparticle–nanobubble solutions with respect to different nanoparticle concentrations (Figure 3.2(b)). It clearly shows that all the experiments (described below) were taking place at pH values far below the pI of amidine nanoparticles,

indicating that charge inversion is not driven by changes in the pH of the mixtures.

Figure 3.2: (a) ζ -potential of 100 nm amidine nanoparticles as a function of pH in 10 mM NaCl at 25 °C. (b) pH measurements of nanoparticle–nanobubble solutions around phase transitions, all pH values are below the isoelectric point of amidine latex nanoparticles (9.5).

3.3 Results and Discussion

3.3.1 Reentrant condensation and inversion of surface charge

Reentrant condensation

Electrostatic interactions are ubiquitous in polyelectrolytes, colloids and biological systems.^{29–32} In mixtures of multivalent ions and macromolecules, a typical feature of phase diagrams is a phase separation regime in which macroion aggregation takes place near the isoelectric point. A subsequent restabilization and redissolution on further increasing salt concentration however takes place. This phenomenon is known as reentrant condensation.^{29, 30, 33, 34}

Figure 3.3: (a) Photograph of 100 nm amidine latex nanoparticle–nanobubble mixture with amidine nanoparticle concentration (1) above C^* , (2) between C^* and C^{**} and (3) after crossing C^{**} . Phase separation occurs between C^* and C^{**} ; (b) Average ζ -potential and (c) apparent hydrodynamic diameter of 60 nm amidine nanoparticle–nanobubble mixtures as a function of amidine nanoparticle concentration.

We now address the interactions between positively charged nanoparticles and nanobubble solutions. Figure 3.3(a) is a photograph of three 100 nm amidine nanoparticle–nanobubble mixture samples at decreasing nanoparticle

concentrations. Note that we do not want to overly dilute the nanobubble solution, as supersaturation would no longer be sustained. Cuvette 1 is a nanoparticle–nanobubble mixture solution with a nanoparticle concentration just above a threshold C^* where the colloids are monodisperse and stable. Cuvette 2 shows a mixture solution with nanoparticle concentration below C^* , where the positively charged latex beads aggregate. Cuvette 3 is a mixture solution with a still lower nanoparticle concentration below a second threshold C^{**} . The aggregates redissolve and the solution becomes stable and monodisperse again.

This observation can be further confirmed by measuring the effective hydrodynamic diameter of the particles in mixtures, as shown in Figure 3.3(c). The figure depicts a typical size evolution of 60 nm amidine latex beads mixed with gas solution at different ratios. The evolution of size starts with a time- and concentration-independent average size in the range of 60 nm to 100 nm above C^* . However, after the concentration passes C^* , a dramatic increase in size occurs ($\sim 1.5 \mu\text{m}$), which is due to the formation of amidine beads aggregates. Once the nanoparticle concentration crosses C^{**} , a sharp transition towards a smaller size, but larger than the original 60 nm nanoparticle size, is observed. This suggests that aggregates disappear and a stable colloidal suspension is restored. Upon further decreasing nanoparticle concentration, the hydrodynamic size does not vary significantly anymore, indicating that, under these conditions, the resulting nanoparticle–nanobubble structures are stable against further aggregation.

Inversion of surface charge

Changes of the surface charge of the nanoparticles, as probed by electrophoresis and expressed as a ζ -potential, were also observed. The ζ -potential of a series of nanoparticle–nanobubble solutions as a function of nanoparticle concentration at 25 °C is presented in Figure 3.3(b). At high

nanoparticle concentration, the nanoparticle–nanobubble solution exhibits a positive charge. Conversely, as nanoparticle concentration decreases, the absolute value of the net charge decreases. The ζ -potential eventually crosses the point of zero charge, at which point the particles become negative. This indicates the formation of negatively charged nanoparticle–nanobubble structures, suggesting bubble nucleation on particle surfaces. This trend eventually saturates and further decreases of nanoparticle concentration do not vary the surface charge significantly. This indicates that positive particles are maximally covered by bubbles nucleated at their surfaces.

A third order polynomial fit was used to extract the point of zero charge C_0 from the overall ζ -potential results. Note that the last two data points in Figure 3.3(b) and 3.3(c) are plotted as open symbols. This is because once the nanoparticle concentration passes below 1×10^9 particles/mL, the DLS instrument is measuring near its sensitivity threshold. Therefore, we use only the data points before 1×10^9 for fitting the point of zero charge. Unsurprisingly, we find that the point of zero charge C_0 always lays between the two phase boundaries of the phase separating regime, i.e. C^* and C^{**} , as seen by comparing Figure 3.3(b) and 3.3(c).

Relation of charge inversion to reentrant condensation

The evidence in Figure 3.3 suggests that charge inversion represents the fundamental mechanism allowing residual attraction to cause agglomeration once electrostatic repulsion vanishes at C_0 . We propose that gas nucleates at the positive particle surface, thus screening part of its overall charge and replacing it with a negative surface. Near neutrality, electrostatic interactions can no longer provide sufficient repulsion, allowing shorter ranged attractive forces to take over. This is the basic trade-off that dictates the stability of most colloids. To what extent the gas bubbles may contribute to these short-ranged interactions remains unclear.

We further systematically extended our experiments to three other different sizes of amidine nanoparticles, i.e., 20 nm, 40 nm and 100 nm. All particles show consistent aggregation and ζ -potential trends with the 60 nm particles (see Appendix for primary data). In particular, we observed reentrant condensation for all sizes tested. The condensation boundaries C^* and C^{**} as well as the concentration at the point of zero charge were extracted for each size. A phase diagram for amidine nanoparticle solution with respect to nanoparticle diameter is shown in Figure 3.4. Three regimes are recognized: regime 1 ($C > C^*$), one liquid phase exists; regime 2 ($C^* > C > C^{**}$), two phases exist; regime 3 ($C^{**} > C$), reentrant solution, the first phase is restored.

Figure 3.4: Phase diagram of amidine latex nanoparticles with respect to nanoparticle sizes. The symbols represent individual samples in the respective regimes.

The apparent size with respect to ζ -potential of all four nanoparticles are presented in Figure 3.5. The different sizes of nanoparticle collapse onto a

single master curve within scatter. The maximum effective diameter of the condensation product for each size is independent of the particle size under the conditions measured. This further proves that reentrant condensation is mainly caused by charge inversion.

Figure 3.5: Hydrodynamic diameter of amidine nanoparticle–nanobubble solutions as a function of zeta potential with different nanoparticle sizes. Different symbols correspond to amidine nanoparticles of the following diameters: $\square$ 20 nm, $\circ$ 40 nm, $\triangle$ 60 nm and ∇ 100 nm.

3.3.2 Influence of size of amidine nanoparticles on reentrant condensation

Concentration at point of zero charge C_o

To further understand the size dependence data in Figure 3.4, we extracted the nanoparticle concentration at the point of zero charge from the ζ -potential data for each particle size (third order polynomial fitting) and the resulting four data points are shown in Figure 3.6.

Based on our hypothesis that reversal of surface charge of particles is due to gas nucleation at the particle surfaces, one could expect that the point

of zero charge is reached when the total surface of the particles reaches a sufficiently small value to allow sufficient coverage by the available gas. The total area available per unit volume of solution, A_{tot} , is given by

$$A_{\text{tot}} = 4\pi R^2 C_o, \quad (3.1)$$

where C_o is the concentration of nanoparticle at the point of zero charge and R is the nanoparticle radius. To simplify analysis, equation 3.1 can be expressed as

$$\ln C_o = a - 2\ln R, \quad (3.2)$$

where a is a constant. The result of a fit to this expression is shown in Figure 3.6 as the red line. The good agreement suggests that the point of zero charge concentration is indeed largely driven by the available surface area under otherwise identical conditions, as indicated by equation 3.2, leading to the trend that condensation occurs at higher concentration for smaller particles.

Figure 3.6: Natural logarithm of nanoparticle concentration at point of zero charge with respect to nanoparticle radius. Concentration at point of zero charge of each size was estimated by a third order polynomial fitting. Four extracted concentrations were then fitted with equation 3.2, see the red curve.

Figure 3.7: Energy barriers of 100 nm amidine–nanobubble solutions calculated from DLVO theory as a function of nanoparticle concentration. The measured zeta potentials were employed as surface potentials of the nanoparticles in the calculation.

3.3.3 Reentrant condensation from a DLVO picture

The phase behavior of reentrant condensation of amidine nanoparticle–nanobubble system can be estimated from DLVO theory.³⁵ This assumes that particles have to overcome an energy barrier in order to aggregate. The attractive van der Waals potential of $U_{\text{vdW}} = -AR/12d$ competes against the electrostatic double layer repulsive potential of $U_{\text{edl}} = (R/2)Ze^{-d/\lambda_D}$, where A is the Hamaker constant, R is the particle radius, λ_D is the Debye length, and $Z = 64\pi\epsilon_o\epsilon(kT/e)^2\tanh^2(e\psi_o/4kT)$ is the electrostatic interaction constant. For a monovalent 1:1 electrolyte e.g. NaCl in our system at 25 °C, $Z = 10^{-10}\tanh^2(\psi_o/103)$, where z is the electrolyte valency of 1 for NaCl, e is the unit charge of 1.6×10^{-19} C, ϵ_o is the vacuum permittivity, ϵ is the relative static permittivity of water, k is the Boltzmann constant, and ψ_o is the potential of the particle surface in mV. We use the measured zeta potential for ψ_o . The energy barrier is calculated as the maximum value (unit kT) of the sum of van der Waals attraction and electrostatic repulsion for each nanoparticle concentration.

Unfortunately, the precise Hamaker constant for our system is unknown. We plot the energy barrier of a series of 100 nm nanoparticle–nanobubble mixture solutions versus nanoparticle concentration in Figure 3.7 for a typical Hamaker constant of $A = 3kT$ for indicative purposes.³⁴ It is seen that the DLVO energy barrier changes in a wide range, from a very high barrier, down towards a very low barrier (close to zero), reentrant to a relatively high barrier at low nanoparticle concentration. This behavior is consistent with phase change behavior in Figure 3.3. While we cannot give an accurate estimate of the exact energy barrier needed to prevent particle–particle collision, the trend agrees surprisingly well with the measured phenomenon of reentrant condensation.

3.4 Conclusion

In summary, we have addressed the interactions between positive nanoparticles and nanobubble solutions. Unlike negatively charged nanoparticles, discussed in Chapter 2, all sizes of positive nanoparticles investigated interact with gas solutions. Furthermore, gas solution can reverse the surface charge of positive nanoparticles via nucleation on their surfaces leading to reentrant condensation, as confirmed by combined electrophoresis (ζ -potential) and size measurements.

When nanoparticle concentration is larger than the threshold C^* and smaller than C^{**} , positive and negative surface charge were observed, respectively, and the solution remains colloidal stable. In contrast, we found that a significant increase in size close to charge neutralization, when nanoparticle concentration passes C^* but prior to C^{**} , between which the condensation occurs. Second, $\ln C_o$ scales linearly to $\ln R$ with a prefactor close to -2 , which supports our hypothesis that reversal of surface charge of positive particles is due to the gas nucleation at their surfaces. Lastly, the evolution trend of estimated DLVO energy barrier matches experimental

observations qualitatively.

We have thus demonstrated for the first time the unique role of gas supersaturated solutions in controlling colloid stability. The observations reported here provide a way to tune the phase transition of colloids initiated by bulk gas solutions and represent a starting point for studying the impact of nanoscale gaseous domains on the charge behavior of positively charged macromolecules.

3.5 Appendix

3.5.1 Reentrant condensation of amidine nanoparticles of other sizes

Figure A3.1: Average ζ -potential and hydrodynamic diameter of amidine nanoparticle–nanobubble mixtures as a function of nanoparticle concentration for a range of particle sizes. Different colors correspond to amidine nanoparticles of the following diameters: black 20 nm, red 40 nm, green 60 nm and blue 100 nm. For all the zeta potential results, a third order polynomial fit method is used to extract the concentration at the point of zero charge.

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INTERACTION OF ANIONIC BULK NANOBUBBLES WITH CATIONIC LIPOSOMES: EVIDENCE FOR REEN- TRANT CONDENSATION

We investigated the interaction of bulk nanobubbles with cationic liposomes constituted by 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC) and anionic liposomes assembled from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG). We employed dynamic light scattering (DLS) and fluorescence microscopy to investigate both the hydrodynamic and electrophoretic properties of the nanobubble/liposome complex. These optical techniques permit directly visualizing the structural change of the bubble–liposome complexes. We observed reentrant condensation with cationic liposomes and gas nucleation with anionic liposomes. This is the first report of charge inversion and reentrant condensation of cationic liposomes induced by bulk nanobubbles.

4.1 Introduction

Interaction of gas bubbles with nanoparticles has been studied both experimentally and theoretically by several authors^{1–3} owing to its relevance in different fields. These range from biomedical applications, such as ultrasound contrast agents in biomedical imaging and drug delivery, to industrial processes that include mineral separation using froth flotation techniques and waste water treatment.^{4–12} However, to our knowledge, no systematic attempt has been devoted to investigating the phenomenology of interactions of bulk nanobubbles with both inorganic and organic nanoparticles and to clarifying the basic mechanism that causes the interaction. Recent investigations by our group of the interaction of gas bubbles with colloidal nanoparticles have led to surprising findings including the formation of bubble–nanoparticle complexes¹³ and reentrant condensation of positive colloidal nanoparticles.

Liposomes, artificial vesicles whose typical sizes range from 20 nm to micrometers in diameter, are closed shells of self-assembled phospholipid bilayers that surround an aqueous core and are employed as model systems for studying the physical properties of biological membranes.¹⁴ Cationic liposomes have recently received much interest as a delivery system for DNA and protein vaccines.^{15–21} They have become a popular gene transfer agent and have been used as an alternative nonviral DNA delivery vector for gene therapy due to their low toxicity, biodegradability, nonimmunogenicity and easy preparation.^{15, 18} However, these nonviral complexes are reported to be rapidly cleared from circulation as a result of enzymatic digestion of plasmid DNA and, in some cases, the phospholipids undergoing oxidation and a hydrolysis-like reaction.^{17, 22, 23} It is a major requirement for cationic liposome-mediated transfection to maintain the colloidal stability of the liposome/DNA complex (lipoplex), which is particularly difficult to achieve at the high DNA concentrations used for *in vivo* studies and clinical trials.^{15, 19} To stabilize liposome/DNA particles formed at high DNA concentration and

thus prolong the circulation time of lipoplexes in blood, polymers such as protamine and poly(ethylene glycol) (PEG) have been used.^{19, 23} Despite these achievements, however, both protamine and PEG are reported to have toxicity effects.^{24–26} Adverse allergic responses to protamines have been reported, including hypotension, bronchospasm, rash, urticaria, cardiovascular collapse and sometimes death.^{27, 28} PEG and PEG-related polymers are often sonicated when used in biomedical applications. Murali *et al.* reported that when sonicated, PEG is very sensitive to sonolytic degradation and PEG degradation products are toxic to mammalian cells.²⁶

Here we investigated the modulation effect of gas nanobubbles on the stability of both cationic and anionic liposomes. The schematic illustration of a single liposome is shown in Figure 4.1(a). Figure 4.1(b) and 4.1(c) show the molecular structures of the phospholipids employed to create cationic liposomes and anionic liposomes, respectively. We present a comprehensive study of the interaction between cationic liposomes and gas nanobubbles by combining dynamic light scattering and fluorescence microscopy measurements. We find that the charge and colloidal stability of cationic phospholipid liposomes can be influenced by gas bubble solutions, the cationic liposomes undergoing a reentrant condensation process upon interacting with nanobubbles. Our initial motivation is that, compared with flexible polymers, gas nanobubbles do not require chemical modification and spontaneously dissipate over time, yet still allow tuning the surface charge of liposomes and shield them from the extracellular environment.

Figure 4.1: (a) Schematic illustration of self-assembled lipid bilayer liposomes in aqueous solution. (b) Molecular structure of cationic phospholipid EDOPC employed to form cationic liposomes. (c) Molecular structure of anionic phospholipid POPG used to create anionic liposomes.

4.2 Experimental Methods

4.2.1 Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG, sodium salt, >99 %) and 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC, chloride salt, >99 %) were purchased from Avanti (Avanti Polar Lipids, Inc. USA) and were used without further purification. NaCl at a 10 mM concentration was prepared using water from Milli-Q system (Millipore, USA) with resistivity 18.2 M Ω .cm at 25 °C. The pH of the sodium chloride solution was not explicitly controlled and had a measured value of 6.5. Texas-Red-modified 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (triethylammonium salt) (Texas Red DHPE) was supplied by Thermo Fisher Scientific and was prepared at a concentration of 0.2 mg/mL.

4.2.2 Liposome preparation via extrusion

All the liposomes were prepared via a mechanical extrusion method. EDOPC and POPG were first dissolved in chloroform forming a clear homogeneous lipid solution at 10 mg/mL. The fluorescence-labeled liposome was produced by mixing target phospholipid with Texas Red DHPE at a mass ratio of 1000 phospholipid to 1 red fluorescent dye, allowing maximum contrast in fluorescent microscope imaging. 99.9 μL of 10 mg/mL stock solution and 4.4 μL of 0.2 mg/mL Texas Red DHPE were transferred into a clean glass vial using glass syringes with a metal needle. The organic solvent was evaporated using a dry nitrogen stream in a fume hood to yield a uniform lipid film. The lipid film was thoroughly dried to remove the residual chloroform by placing the glass vial under vacuum for one hour. The dry lipid film was then resuspended in 1 mL NaCl aqueous solution (10 mM) to a final lipid concentration of 1 mg/mL, and vortexed for ~ 1 min above the phase transition temperatures T_c of the lipids (-2°C for POPG and -17°C for EDOPC). The resulting lipid suspension was extruded eleven times through 0.1 μm polycarbonate membranes using a Mini Extruder (Avanti Polar Lipids, Inc. USA). Finally, a homogeneous liposomal suspension of uniformly sized unilamellar vesicles with an average diameter of 116 ± 8 nm (nominally 100 nm) was obtained. The final solution was wrapped in aluminum foil and kept in the dark in a refrigerator at 4°C .

4.2.3 Nanobubble generation and characterization

The nanobubble solutions were generated via high power water electrolysis in a cell consisting of planar Pt electrodes with an area of $\sim 66\text{ cm}^2$. 10 mM NaCl aqueous solution was pressure-driven through the cell at a flow rate of 500 mL/min and treated with a cell voltage of 24 V and an average current of 3.0 A. As a result, water was decomposed into oxygen and hydrogen gas which became dissolved in the water stream. Through this process, the solution

becomes supersaturated with oxygen and hydrogen gas. Stable nano-sized colloidal objects, commonly referred to in the literature as nanobubbles,^{29–31} were observed in the supersaturated solutions following water electrolysis.

Characterization of the nanobubble solution was done by dynamic light scattering (DLS) for sizing and electrophoresis for zeta potential determination, both conducted at 25 °C with the help of a Malvern Zetasizer Nano ZS equipped with a laser (633 nm) set at an angle of 173°. Detailed operating parameters of each technique were selected for consistency with earlier work.¹³ In short, the average and standard deviation of 5 measurements were computed for further analysis. Measurements of our nanobubble solutions exhibited a peak size and full width at half maximum of 223 nm and 94–529 nm, respectively, and a negative charge with a mean zeta potential of -19 ± 3 mV.

4.2.4 Particle size and zeta potential determination

All the measurements were conducted in 10 mM NaCl (pH 6.5) as a reference system. Dynamic light scattering and electrophoresis measurements were once again applied for determination of hydrodynamic diameter and zeta potential of the liposome/bubble mixtures, respectively. The signal analysis was performed using software provided by the manufacturer (Zetasizer Software, Malvern). The interaction of anionic nanobubbles with liposomes was initiated by adding nanobubble solution to an existing liposome suspension to a final volume of 1 mL, followed by gentle mixing and ~ 1 min ultrasonication.

4.2.5 Fluorescence microscopy imaging

Fluorescent microscope (Olympus, IX71) equipped with a powerful 120 W lamp (X-Cite 120PC Q) as an excitation light source and a digital camera

(Olympus, DP70) for image acquisition was used to visualize and image the structure of nanobubble/liposome complexes at several representative EDOPC concentrations. The Texas Red-labeled samples were exposed to a laser with an excitation wavelength of 595 nm using a filter cube (Olympus, IX2-RFAC). Depending on the lipid concentration, the laser intensity was adjusted to achieve maximum contrast in fluorescent microscopy imaging.

4.3 Results and Discussion

4.3.1 Reentrant condensation of cationic liposomes with nanobubbles

Colloidal stability of liposomes is a primary requirement for cationic liposome mediated gene transfection.³² Therefore, we used dynamic light scattering to assess both the hydrodynamic size distribution and electrophoretic properties of bubble/liposome complexes mixed at a series of lipid concentrations ranging from 0.1 mg/mL to 1 ng/mL, as presented in Figure 4.2. The average size of nanobubble/EDOPC complexes remained essentially constant (that is, on the order of 200 nm) until a critical coalescence concentration (C^*) of 0.5 $\mu\text{g/mL}$ was reached. Below this concentration a gradual increase in size was observed up to a concentration of 0.1 $\mu\text{g/mL}$, at which point, coalescence of complexes occurred, leading to structures larger than 1 μm in diameter. As the lipid concentration was further decreased, a remarkable transition back toward smaller size was observed, where the increase in the relative concentration of gas solution started to restabilize the large aggregates. Once the lipid concentration reached a second critical coalescence concentration (C^{**}) of 0.03 $\mu\text{g/mL}$, the complex size became once again essentially constant at a value close to that at high lipid concentration. This indicates that, upon further decreasing the lipid concentration, the colloidal stability of EDOPC complexes was restored. Note that once the lipid

concentration was decreased down to $0.01 \mu\text{g/mL}$, the measurements were near the detection threshold of the DLS instrument which may introduce some systematic error (to emphasize this, the last two data points are plotted as open symbols).

Figure 4.2: (a) Average zeta potential and (b) hydrodynamic diameter of bubble/liposome (100 nm) complexes as a function of EDOPC concentration. Size determination was performed after 1 min of ultrasonication at room temperature. C^* and C^{**} are two critical coalescence concentrations (CCC) separating the graph into three different regimes and were determined empirically.

Based on earlier measurements with positively charged nanoparticles (Chapter 3), it may be expected that the presence of nanobubbles influences the charge state of cationic liposomes. To test this hypothesis we performed zeta potential measurements, a quantity that is directly related to the surface charge of the particles. Figure 4.2 shows the average zeta potential of nanobubble/EDOPC complexes as a function of lipid concentration. A clear inversion of the surface charge from positive to negative is observed upon decreasing the cationic liposome concentration. That is, the point of (effective) zero charge ($\sim 0.1 \mu\text{g/mL}$) lies in between the critical coalescence

concentrations C^* and C^{**} . The presence of large particles is presumably due to liposome coalescence. This indicates that the effect of the nanobubble solution is to neutralize the surface charge of the cationic liposomes, presumably due to nucleation at the liposome surface,¹³ leading to a loss of colloidal stability and coalescence of the liposomes into large aggregates.

We thus find that nanobubble/liposome complexes exhibit a three-regime model of colloidal stability, as shown in Figure 4.2, in which regimes 1 and 3 are characterized as highly positive and negative colloidal stable bubble/EDOPC systems, respectively, whereas regime 2 corresponds to colloiddally unstable bubble/liposome aggregates. This phenomenon is known as reentrant condensation.^{33, 34}

Figure 4.3: Control experiments: (a) average zeta potential and (b) hydrodynamic diameter of salt/liposome mixtures as a function of lipid concentration.

To further confirm that coalescence is indeed due to nanobubble/liposome interactions, we performed DLS and zeta potential measurements on control

4

samples where the liposome solution was mixed with an untreated 10 mM NaCl solution. All other parameters were kept the same as the measurements with hydrolysis-treated electrolyte presented above. The results are shown in Figure 4.3. Contrary to nanobubble/EDOPC mixtures, the particle size remained essentially constant (on the order of 200 nm) and no agglomeration occurred for salt/EDOPC. The zeta potential remained positive over the concentration range where the nanobubble solution exhibited reentrant condensation. The decline of zeta potential with decreasing lipid concentration is a suspected experimental artifact.^{35–37} Tantra *et al.* observed a shift in zeta potential values to less negative values for nanoparticle suspensions at extreme dilution and they attribute it to an increase in signal arising from extraneous particulate matter.³⁵

To further elucidate the interaction between nanobubbles and cationic liposomes, the structure of the nanobubble/liposome complexes at several representative EDOPC concentrations was visualized by fluorescence microscopy. Figure 4.4(a) shows the fluorescence image of the 1 mg/mL source solution of cationic liposomes before mixing with nanobubble solution. A nearly uniform red background is observed, which is due to the large concentration of out-of-focus fluorescently labeled liposomes. A few bright spots are also observed, corresponding to individual liposomes in the focus plane of the microscope. These spots are resolution limited, therefore the size of the liposomes cannot be inferred from these fluorescence images. By gradually decreasing the cationic liposome concentration via mixing with nanobubble solution, the uniform background fades (Figures 4.4(b)) until only discrete entities could be discerned against a dark background (Figures 4.4(c)–(e)). Below the critical coalescence concentration of C^* (Figure 4.4(d)), objects larger than the resolution limit begin to appear, increasing further in size at lower concentrations (Figure 4.4(e)). This behavior is consistent with the DLS measurements, which exhibit a marked increase in hydrodynamic diameters in the same concentration range. In contrast, control measurements in which untreated NaCl solution is used instead of nanobubble solution to dilute

Figure 4.4: Fluorescence microscopy of nanobubble/EDOPC liposome complexes labeled with Texas Red dye at representative lipid concentrations. (a) Source EDOPC liposome solution with a concentration of 1 mg/mL before interacting with nanobubbles. The uniform red background is due to contributions from out-of-focus liposomes in this high concentration solution. (b), (c) Bubble/EDOPC complexes at lipid concentrations before critical coalescence concentration C^* . (d) Nanobubble/EDOPC complexes at lipid concentration near C^* . (e) Nanobubble/EDOPC complexes at lipid concentration of 0.1 $\mu\text{g/mL}$, at which significant coalescence occurred. The scale bars represent 5 μm . In (c)–(e), the density of spots was too low to allow visualizing several in a single frame. Therefore three representative images of individual complexes are shown. In each case the bottom right panel (green) is a control experiment in which EDOPC liposomes were mixed with nanobubble-free solution. The observed spots are resolution limited except in panels (d)–(e). The intensity scale at each concentration has been re-scaled for clarity and intensities thus cannot be directly compared.

the liposome solution show no such aggregation behavior (green images in Figures 4.4(c)–(e)). The control measurement in Figure 4.4(e) shows only a black background. This is because at a low lipid concentration of $0.1 \mu\text{g/mL}$, it is practically impossible to capture the objects. The aggregates, on the other hand, are large and diffuse more slowly, and therefore can be manually tracked. For the same reason, systematic fluorescence measurements at concentrations below C^{**} proved impractical due to the rarity of the events.

Taken together, the phenomenology of reentrant condensation of cationic liposomes can be summarized in a charge inversion scenario similar to our previous work on positive amidine nanoparticles. That is, we propose that the supersaturated solution causes the formation of gas bubbles on the surface of the liposomes, screening out the positive liposome surface charge and exhibiting a negative surface charge to the solution. Near the point of zero charge this renders the colloidal suspension unstable. Further decreasing the lipid concentration causes the net surface charge to reverse sign which is again colloidally stable.

4.3.2 Interactions of nanobubbles with anionic liposomes

We also looked at the interaction between nanobubbles and anionic liposomes in the same concentration range as for cationic liposomes. As shown in Figure 4.5, the zeta potential of nanobubble/DOPG complexes remained negative with a gradual decline in magnitude (from very negative to less negative, Figure 4.5(a)) whereas the size slightly increased (Figure 4.5(b)), consistent with a gas layer nucleating onto liposome surface. This behavior is highly reminiscent of our earlier measurements on gold nanoparticles in Chapter 2, which were interpreted as resulting from bubble nucleation on the nanoparticle surface.

In this interpretation, the decrease in magnitude of the zeta potential occurs because the bubbles are less negative than the anionic liposomes. They

Figure 4.5: Interaction of nanobubbles with anionic liposomes: (a) average zeta potential and (b) hydrodynamic diameter of nanobubble/POPG complexes as a function of POPG concentration.

can thus shield the strong negative charge of the anionic liposomes, resulting in a decrease of net surface charge for the bubble/DOPG complexes. These results are thus again consistent with our hypothesis that nanobubble nucleation at the liposome surface accounts for the bubble–liposome interaction.

4.4 Conclusion

We have presented the first experimental observations of reentrant condensation of model cationic liposomes in bulk solution under the influence of anionic nanobubbles using both microscopy and size measurements. Zeta potential measurements indicate that this coincided with surface charge inversion. Based on the observations, we propose a mechanism of gas nucleation on the liposome surfaces to address the bubble/liposome

interactions. Bubbles nucleate on the liposome surfaces and thus screen their surface charge, leading to a shift or even reversal of the sign of the zeta potential. These observations provide a new pathway to tune liposome interactions in solution. Further studies are needed to establish whether these results can be generalized to vesicle separation techniques and delivery systems. From the medical application point of view, for example in blood, usefulness depends on how fast it takes the nanobubbles to dissipate. We currently lack kinetic data for our nanobubbles, further studies are needed to address this issue. This is of particular interest for the growing number of studies that isolate and manipulate liposomes, providing a new mechanism that does not require chemical modification, and by which they could be redissolved afterwards.^{38, 39}

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SELF-ASSEMBLY OF VIRAL CAPSID PROTEINS DRIVEN BY COMPRESSIBLE NANOBUBBLES

Colloidal nanobubbles (NBs) were observed in gas-saturated aqueous solutions following high power water electrolysis. The self-assembly properties of viral capsid protein (CP) influenced by nanobubble solutions were investigated. Interestingly, we found that gas solutions were able to work as a negative template to trigger the self-assembly of capsid proteins of cowpea chlorotic mottle virus (CCMV) in the absence of the viral genome. The process was demonstrated by three distinct techniques including dynamic light scattering (DLS), atomic force microscopy (AFM), and transmission electron microscopy (TEM). Additionally, NB-induced self-assembly of viral capsid proteins was protein-concentration dependent. Low CP concentrations led to assembly of 18 nm (comparable to $T = 1$ virus capsids) virus-like particles (VLPs) whereas high CP concentrations led to 28 nm (similar to $T = 3$) VLPs.

5.1 Introduction

Small bubbles such as bulk nanobubbles (that is, with a diameter less than 10^{-6} m) have been observed in gas saturated solutions following high power water electrolysis.^{1–3} Bulk nanobubbles can be detected by a number of different techniques including total internal reflection microscopy (TIRM),⁴ resonant mass measurements,⁵ transmission electron microscopy (TEM),⁶ Cryo-EM,⁷ and light scattering,^{2, 8} the latter being most prominent. These bubbles exhibit several properties that differ from macroscopic bubbles (diameter larger than 10^{-3} m), such as lower buoyancy which translates in longer times to equilibrate with a gas phase and negative zeta potential which inhibits the agglomeration or coalescence of bubbles.⁹ In addition, small bubbles show large specific interfacial area, by virtue of which, we expect an efficient interactions of bubbles with objects added to the solution. We recently reported that gas bubbles both interact with negatively and positively charged nanoparticles via nucleation on the nanoparticle surfaces (Chapter 2 and Chapter 3), particularly, it causes reentrant condensation of positive particles upon mixing with gas saturated solutions.

Self-assembled viral protein cages have attracted great interest because of their controlled assembly and disassembly properties as well as their potential as biomolecular scaffolds with a highly organized structure.¹⁰ Spherical viruses are mostly composed of a few hundreds subunits that self-assemble to encapsulate their genome for storage and transport. However many viral capsid proteins (CPs) can be driven to assemble even without their natural cargo.¹¹ In this respect, a range of applications, varying from drug delivery to nanoreactors among which CPs are assembled and designed as cargo carriers have been explored extensively.^{11–14} In this work, we performed the controlled assembly using cowpea chlorotic mottle virus (CCMV)-based viral capsid proteins.

CCMV is a member of the *Bromoviridae* family of plant viruses with an

icosahedral shell of 28 nm of outer diameter and 18 nm of inner diameter, which surrounds a central core of single-stranded viral RNA.¹⁵ A particular interesting characteristic of CCMV, compared with other spherical viruses such as cowpea mosaic virus (CPMV), is that the protein shell consisting of 180 identical subunits of approximately 20 kDa with Casper Klug triangulation number $T = 3$,^{16, 17} can reversibly disassemble into 90 capsid protein (CP) dimers *in vitro* at high pH and high ionic strength. Depending on the pH and salt concentration, CCMV is able to reassemble into empty VLPs and other different capsid protein polymorphs.¹⁸ For example, after removal of RNA, empty capsids of the same size and geometry as the native virus are reassembled if the pH is reduced to 5. Furthermore, unlike adenoviruses, CCMV is nonpathogenic and is considered as a promising molecular platform for the targeted delivery and treatment of many human diseases.¹⁹ At neutral pH, CP assembly requires a polyanionic template such as negative inorganic nanoparticles and synthetic anionic polymers.^{10, 11, 20} These templates can self-assemble with CCMV CPs that, depending on medium ionic strength and template, can produce varieties of structures such as tubes and icosahedral capsids with $T = 1$ (containing 30 CP dimers), $T = 2$ (60 dimers) and $T = 3$ (90 dimers) structure.^{10, 18, 21} In this respect, CCMV offers the versatility to accomplish nanoassemblies of different sizes and nanostructures.

So far, inorganic nanoparticles of various types and functions have been widely used as templates to grow VLPs,^{10, 11, 22} but, to the best of our knowledge, a metastable soft template like a nanobubble has never been used to template the growth of VLPs. Our DLS, AFM and TEM results indicate that, at neutral pH, nanobubbles generated by high power water electrolysis template the CP assembly. Importantly, under the controlled pH and ionic strength, the size of CCMV-based CP assemblies is protein concentration dependent, which holds potential as a new route for the design of desired VLPs. Besides, understanding the CP-NB system could also lead to a better understanding of nanobubble characteristics.

5.2 Experimental Methods

5.2.1 CCMV capsid protein dimer isolation

1 mL of wild-type CCMV (~ 10 mg/mL) was purified from infected cowpea plants and stored in virus buffer solution (0.1 M sodium acetate, 0.001 M disodium EDTA and 0.001 M sodium azide, pH 5.0) at 4 °C in accordance with a well-established protocol.^{23, 24} The purified CCMV suspension was dialyzed against a total volume of 1000 mL RNA buffer (50 mM Tris, 500 mM calcium chloride and 1 mM dithiothreitol, pH 7.5) in two steps: a first step for 2 hours with 200 mL followed by 800 mL overnight at 4 °C. This resulted in a white precipitate. Ultracentrifuge with a speed of 40000 rpm (Sorvall, F50L-24×1.5 rotor) was applied for 2 hours to centrifuge down the white precipitate containing RNA. The supernatant (~ 1 mL) was dialyzed 3 times against a total volume of 1000 mL clean buffer (50 mM Tris, 500 mM sodium chloride, 1 mM dithiothreitol, pH 7.5), twice for 3 hours with 330 mL followed by 330 mL overnight. The resulting solution was analyzed by UV-Vis absorption at 280 nm (Thermo Scientific NanoDrop 1000 UV-Vis spectrophotometer) to determine the concentration of CP. Finally, the isolated CP dimer solution was dialyzed against 50 mM Tris buffer (300 mM sodium chloride and 5 mM magnesium chloride, pH 7.2) before application for VLP assembly.

5.2.2 Nanobubble generation

The generation of nanobubbles was performed in accordance with Chapter 2 of this thesis. Briefly, 10 mM sodium chloride solution was prepared with milli-Q water. The nanobubble solutions were then created by high power water electrolysis. The salt solution was delivered at the one side of the flow cell, flowed through the plate electrodes being electrolyzed simultaneously, finally exited at the other side of the flow cell. This water electrolysis process

decomposes water into oxygen and hydrogen gas, both are allowed to dissolve back into the water stream, which makes the solution supersaturated with oxygen and hydrogen gas. The electrolysis was treated with a voltage of 24 V and an average current of 3 A at a flow rate of 500 mL/min.

5.2.3 Characterization of nanobubble solutions

Characterization of the nanobubble solution was carried out by dynamic light scattering (633 nm laser, scattering angle of 173°, Zetasizer Nano ZS, Malvern Instruments) for sizing, electrophoresis for zeta potential (Zetasizer Nano ZS, Malvern Instruments) and nanoparticle tracking analysis (642 nm laser, NTA, NS500, Nanosight, Malvern Instruments) for concentration, all being implemented at 25 °C. Each sample was used for five measurements and each measurement took 60 s. The average and standard deviation of 5 measurements were used for further analysis.

DLS gives particle size in terms of hydrodynamic diameter. Nanobubbles exhibited a single peaked distribution, with peak diameter and full width at half maximum of 223 nm and 94–529 nm, respectively, as shown in Figure 5.2(a). The solution exhibited long term stability on the scale of days. Additionally, we measured the nanobubbles to be negatively charged with a mean zeta potential of 5 measurements of -20 ± 3 mV.

The concentration was directly measured by counting the number of tracked particles in a known volume using NTA. Our nanobubble solution had a concentration of $\sim 10^7$ – 10^8 /mL.

5.2.4 Nanobubble-induced self-assembly of VLPs

The above mentioned nanobubble solutions were used to trigger the self-assembly of VLPs with CPs. In a typical experiment, 400 μ L of nanobubble

solution was quickly mixed with 200 μL of pre-purified capsid protein solution (1–5 mg/mL of protein). This mixture was mechanically mixed for 1 minute and was incubated overnight at 4 °C with gentle mixing to allow the formation of VLPs. For control experiments, 400 μL of the same stock NaCl solution but which was not subjected to electrolysis was mixed with 200 μL of CP solution under the same conditions.

5.2.5 Characterization of VLP solutions

The size of the VLPs was first characterized by dynamic light scattering using a Microtrac Nanotrac Wave W3043 (Anaspec) instrument at 25 °C with the laser wavelength of 780 nm and a scattering angle of 90°. Microtrac FLEX Operating software was employed to record and analyze the data. Viscosity and refractive index of the buffer were presumed to be identical with water. In terms of refractive index of VLPs, that of native CCMV $n = 1.54$ was used. All measurements were performed in triplicate.

In addition, the VLPs were imaged by atomic force microscopy (AFM, Asylum Cypher, Santa Barbara, CA, USA) with a silicon cantilever in air using AC tapping mode with blueDrive photothermal excitation. The spring constant and the tip radius of curvature were quoted to be 1.7 N/m and 7 nm by the manufacture (Olympus Japan), respectively. Samples were drop-casted on the silicon wafers which were pre-modified with 3-(aminopropyl)triethoxysilane (APTES). Statistical analysis of the AFM image was achieved by extracting the height value of all the pixels that form a complete particle. The pixel with the highest height value was then used to determine the height of that particle.

Finally, the VLPs were also imaged with transmission electron microscopy (TEM) on a FEG-TEM (Phillips CM300ST-FEG) operated at 300 kV acceleration voltages. TEM samples were prepared by drop casting 5 μL of VLPs sample onto a 200 mesh copper grid for 2 min prior to removal of excess

solution by using a sterile paper. Next, samples were negatively stained by adding 5 μL of 1% (w/v) uranyl acetate onto the grid for 30 s and removed afterwards to provide the best contrast. The diameter of imaged VLPs were measured using ImageJ software to determine the size distribution. The size of each particle was measured in two orthogonal directions.

Figure 5.1: Schematic representation of nanobubble triggered self-assembly of virus-like particles (VLPs) with viral capsid proteins (CPs). Low CP concentration leads to assembly of 18 nm VLPs (similar to $T = 1$, image does not represent the real structure), whereas high CP concentration leads to assembly of 28 nm VLPs (similar to $T = 3$).

5.3 Results and Discussion

5.3.1 Characterization of self-assembled VLPs driven by compressible nanobubbles

As discussed, capsid assembly *in vitro* is either induced by RNA at neutral pH or spontaneous at low pH ($\text{pH} \leq 5$) without RNA. At acidic pH, the interactions between dimers results in a globally stable structure; a neutral pH, empty capsids dissociate. *In vitro* assembly at neutral pH is not specific

for viral RNA, and can also be induced by other polyanions or even negatively charged templates. Our goal of this study is to test whether nanobubbles can be used as a template to trigger the formation of VLPs. To accomplish this goal, we first focused on monitoring the CP assembly using different techniques.

The experimental route for nanobubble-induced assembly of VLPs is presented in Figure 5.1. Wild-type CCMV was purified from infected vigna unguiculata plants (cowpea) and stored in virus buffer solution (0.1 M sodium acetate buffer, 0.001 M disodium EDTA and 0.001 M Sodium azide, pH 5.0) at 4 °C in accordance with a well-established protocol.^{23, 24} The virus dissociated into RNA and protein subunits when it was dialyzed against 0.5 M Ca₂Cl at pH 7.5.²⁵ Isolated capsid protein dimers were finally stored in 50 mM Tris buffer (300 mM sodium chloride and 5 mM magnesium chloride, pH 7.2). Nanobubble-triggered self-assembly of VLPs was achieved by adding freshly generated nanobubble solutions into capsid proteins in a 2 : 1 (v : v) ratio and incubated overnight (see Experimental Methods).

We first examined the assembly of CPs in the presence of a negatively charged nanobubble solution ($\zeta = -23 \pm 7$ mV, pH = 6.5) with dynamic light scattering (DLS). We hypothesized that nanobubbles with size around 200 nm (Figure 5.2(a)) was most likely to yield similar sized VLPs, as widely reported using incompressible templates.^{10, 14, 22} Instead, interestingly, the nanobubble solution triggered self-assembly of CPs (3.2 mg/mL, in Tris buffer, pH = 7.2) of VLPs with a diameter of 26 nm, which is similar to the native CCMV virus (Figure 5.2(b) and 5.2(c)). In contrast, in the control experiment, mixing with an untreated 10 mM NaCl solution yielded no VLPs (Figure 5.2(d)), indicating that indeed the self-assembly of CPs into VLPs at neutral pH and low ionic strength (pH 7.2, 110 mM NaCl) was driven by nanobubbles.

Both AFM and TEM imaging were used to verify the formation of VLPs (Figure 5.3). As depicted in Figure 5.3(a), a considerable number of VLP assemblies could be imaged by AFM. The histogram shown below

Figure 5.2: Hydrodynamic diameter of (a) pure nanobubble solution, (b) native CCMV virus solution, (c) self-assembled VLPs induced by nanobubbles (NBs) and (d) blank (CPs mixed with an untreated 10 mM NaCl solution). All other parameters were kept the same throughout.

(454 counts) revealed a mean diameter of 27 nm, which matched well with the hydrodynamic VLP diameter from DLS. The same sample was also analyzed by TEM. Similar to both DLS and AFM results, we again observed assembly of VLPs with a mean diameter of 30 nm (463 counts, Figure 5.3(b), bottom). Given the identical results from three distinct techniques, this strongly suggests that self-assembly of CPs into VLPs is induced by compressible nanobubbles.

5.3.2 Self-assembly is controlled by the amount of gas

Since VLP formation is induced by the nanobubbles, it is anticipated that the removal of gas from the bubble solution may be accompanied by a drop in

Figure 5.3: Analytical data of nanobubble induced self-assembly of virus like particles. (a) Atomic force microscopy image of VLPs and their size distributions below (454 particles were counted). (b) Negatively stained transmission electron microscopy image of VLPs and their size distributions below (463 particles were counted).

efficiency or even failure of self-assembly of VLPs. To demonstrate that the nanobubbles are responsible for the self-assembly of VLPs at neutral pH and low ionic strength, we investigated whether CPs lacking negatively charged nanobubbles could self-assemble into VLPs under the same conditions. As shown in Figure 5.4(a), when nanobubble solution was degassed by a vacuum pump (7.0 mbar, MZ 2C NT, VACUUBRAND) for 3 hours, the size distribution peak at 26 nm exhibited relatively low intensity and coexisted with another size peak located at 1 nm. When the nanobubble solution was degassed for a longer period (5 hours), only a single size distribution at 1 nm corresponding to pure CP solution was observed (Figure 5.4(b)), which

is highly consistent with the DLS result for the control with a CP-NaCl mixture (Figure 5.2(d)). Combining those two observations together clearly demonstrates that removal of gas from bubble solution leads to the failure of assembly of VLPs and gas bubbles play an important role in VLP self-assembly process.

Figure 5.4: Blank: dynamic light scattering measurement of self-assembled VLPs induced by degassed nanobubbles. The nanobubble solution was degassed for (a) 3 hours and (b) 5 hours prior to mixing with CPs.

5.3.3 Self-assembly of VLPs is CP concentration dependent

The structure and stability of the polymorphs of CCMV-based CP assembly can be comprehended in terms of electrostatic interactions and the way they affect the spontaneous curvature of protein networks.^{26–28} There are two types of interactions within the protein particle: the interaction between neighboring CPs (CP-CP) and the interaction between CP and cargo (CP-cargo). The entire assembly process depends on the strength of CP-CP attraction relative to CP-cargo attraction. CP-CP interactions come from a combination of hydrophobic attraction and electrostatic repulsion and are controlled by pH. At high pH, the electrostatic repulsion between the negatively charged CPs overtake the hydrophobic interactions between CPs which stabilize the CP solutions. Therefore, assembly the empty capsids is

5 favored by lowering the pH. Indeed, it has been reported that raising the pH above 6.5 destabilizes CP-CP interactions and causes capsid swelling,²⁶ whereas lowering the pH to 4.8 reduces the electrostatic repulsion between CPs and allows the hydrophobic attraction to become dominant and drive the assembly.²⁷ CP-cargo interactions are more sensitive to ionic strength. The basic residues at the N-terminus of CP have high pK_a s so that they remain positively charged under the pH we studied. Thus electrostatic attractions between positively charged N-terminus of CP and negatively charged cargo are modulated by ionic strength and their binding is favored by low ionic strength. Therefore, it can be stated that electrostatic interactions of CP-NB is the key force drives the self-assembly of VLPs at neutral pH and low ionic strength (pH 7.2, 110 mM NaCl).

To study CP-NB interactions, we featured the role of CP concentration in the assembly. We characterized the assembly of VLPs with different CP concentrations while keeping the pH and ionic strength of the mixture (pH 7.2, 110 mM NaCl) and zeta potential of nanobubbles (-20 mV) constant. First, when 1 mg/mL CP was mixed with nanobubble solution at regular volume ratio 1 : 2, the NB-CP formed VLPs with a peak diameter of 18 nm, suggesting a similar to $T = 1$ structure (Figure 5.5 red curve). Increasing concentration of CP to 2 mg/mL, we observed assembly of VLPs with a peak diameter of 21 nm, which is closer to the diameter of the $T = 2$ structure (Figure 5.5 orange curve). Further increasing CP concentration up to 3 mg/mL, 4 mg/mL and 5 mg/mL, a peak diameter of 26 nm, 27 nm and 30 nm was obtained, respectively, similar to the $T = 3$ structure (Figure 5.5 yellow, green and blue curve, respectively). These results suggest that the size distribution of VLPs reflects the assembly mechanism with the optimal size being dictated by the charge ratio of CP and nanobubbles. No self-assembly products were obtained with CP concentrations below 1 mg/mL, presumably due to the low CP supply.

To summarize the above, self-assembly of VLPs induced by nanobubbles is CP concentration dependent and the concentration ratio of CP to NB controls

the resulting structure of VLPs.

Figure 5.5: Size distribution of self-assembled VLPs as a function of CP concentration. At 1 mg/ml, VLPs of 18 nm diameter (similar to $T = 1$) were assembled, whereas VLPs of 28 nm diameter (similar to $T = 3$) were assembled with CP at 5 mg/ml. The crossover occurred for CP concentration between 1 mg/ml and 5 mg/ml.

However, electrostatic interactions are not the only important contributor to VLP assembly in the CP-NB system. While we cannot yet describe the exact pathway for CP-NB assembly into the Kaspar Clug symmetry, the observations suggest that NBs facilitate the nucleation of VLPs. This could occur via the growth of a single proto-VLP or by driving the formation of hexameric and pentameric subunits which then combine.

Figure 5.6: Size distribution of self-assembled VLPs triggered by nanobubbles of different zeta potentials. We tested NBs with a zeta potential of -13 mV (red curve) and -20 mV (blue curve). CPs tend to form more stable VLPs when driven by more negatively charged nanobubbles.

5.3.4 Self-assembly of VLPs is charge dependent and reversible

To further verify that CP-NB interaction is the main reason for self-assembly of VLPs, we carried out a competition study to examine whether the amount of charge of NB affects the assembly, as shown in Figure 5.6. We found that for NBs at a zeta potential of -13 mV, most of CPs remain disassembled, whereas for NBs at zeta potential of -20 mV, most of CPs self-assembled into VLPs with a diameter of 30 nm, again suggesting an architecture similar to $T = 3$. The inability of CPs to assemble at zeta potential of -13 mV indicates that the lack of charge matching between N-terminus of CP and negatively charged NB lowers the attractive forces of CP-NB, thus lowering the VLP

formation efficiency. This is reminiscent of the encapsulation of genomic material, as a universal charge ratio of 1.6 for genome to total capsid is needed for most RNA viruses.^{28–30}

Another observation supporting NP-NB interaction is that assembly of VLPs is reversible (Figure 5.7). Figure 5.7(a) shows that after a week of VLP assembly, the capsid solution started to become unstable and a size distribution below 10 nm corresponding to hexameric or pentameric subunits appeared. However, after adding an extra amount of negatively charged nanobubble solution (400 μ l) into the unstable capsids, the disassembled CP reassembled back into intact VLPs (Figure 5.7(b)). This observation demonstrates the importance of the role of charge played in the assembly process and that the assembly is reversible when the NB solutions become less saturated.

Figure 5.7: The self-assembly of CP into VLPs driven by nanobubbles was reversible. Samples which disassembled after a week and contained free precursor proteins (a) could be reassembled back into VLPs (b) by the addition of NB solution.

5.4 Conclusion

In conclusion, a simple route for the formation of CCMV-based VLPs driven by compressible nanobubbles via CP-NB attractions at neutral pH

and low ionic strength (pH 7.2, 110 mM NaCl) was presented. Three distinct characterization techniques were employed to demonstrate the successful assembly of VLPs. This assembly was found to be CP concentration dependent. At low CP concentrations, CP favored the assembly of 18 nm, similar to $T = 1$ VLPs. Further increasing CP concentrations, the capsid formed a structure closer to pseudo $T = 2$ and even the native $T = 3$ capsid symmetry if the concentration was above 3 mg/mL. Furthermore, the assembly of VLPs could be attenuated by NB surface charges and the self-assembly of VLPs was reversible. Controlling the assembly of a virus capsid with compressible nanobubbles enables the formation of well-defined protein architecture induced by a distinct new template, whose size and geometry does not need to correspond to the shape and size of the final product. This help minimize the complexity of the assembly process.

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SUMMARY

Bulk nanobubbles are nanoscopic bubbles formed in aqueous solution by supersaturation and have been discovered to form stable colloidal suspensions. They are attractive due to their relevance for many applications ranging from industrial processes such as cleaning, froth flotation and wastewater treatment, to biomedical fields that include ultrasound contrast agents, drug delivery and disease diagnosis. However, studies into these applications are partial, scattered and non-systematic. This thesis attempts a systematic study of interactions of bulk nanobubbles with a series of soft matter systems, which includes various colloidal suspensions, liposomes and viral capsid proteins. Our study also presents a range of experimental results and controls to improve the understanding of nanobubble features.

The thesis starts by giving a brief literature review in Chapter 1 about bulk nanobubbles, their generation, characterization methods as well as possible stability mechanisms and potential applications. Despite the promise of practical implications, we attempt to build up the bridge to investigate their interactions with soft matter systematically.

For the first investigation described in Chapter 2, we looked into the

interaction of bulk nanobubbles with negatively charged Au nanoparticles. The resulting Au nanoparticle–nanobubble interaction suggests a scenario of nucleation and growth of nanobubbles directly on nanoparticles. This is supported by the fact that the smaller Au nanoparticles do not interact with nanobubbles but larger nanoparticles interact strongly. If collisions are the driving force for interaction, we would instead expect the smallest nanoparticle to interact the strongest due to both the lowered DLVO energy barrier and the increased diffusive speed.

As a next step, we explored the interaction with positively charged amidine polystyrene beads over a range of sizes as reported in Chapter 3. We observed reentrant condensation of amidine nanoparticles of all sizes upon mixing with bulk nanobubble solution. This behavior is directly monitored through the size change and reversal of electrophoretic mobility of the mixtures. By studying the relation between nanoparticle concentration at the point of zero charge and the total particle surface area, we gained a further understanding of the nucleation mechanism underlying our observations.

In Chapter 4 we moved onto liposomes in bulk solutions in order to investigate the phenomenology of interactions of bulk nanobubbles in an organic system. We employed dynamic light scattering to probe both the hydrodynamic and electrophoretic properties, and fluorescence microscopy to visualize the structure change of nanobubble–liposome complexes. The same reentrant condensation was observed with cationic liposomes, further supporting the interaction mechanism of gas nucleation on the liposome surfaces.

Last but not the least, the thesis ends up with studying the bulk nanobubble-induced self-assembly of viral capsid proteins disassembled and purified from native CCMV. In Chapter 5, we address the successful self-assembly of capsid proteins by three distinct techniques including dynamic light scattering, atomic force microscopy, and transmission electron microscopy. In this process, bulk nanobubbles play a role as templates. We

also found that depending on capsid protein concentrations, the same building blocks are able to assemble into different capsid sizes corresponding to different building structures.

SAMENVATTING

Bulk nanobubbels zijn nanoscopische bubbels die ontstaan in een waterige oplossing door oververzadiging. Ondekt is dat deze bubbels stabiele colloïdale suspensies vormen. Ze zijn aantrekkelijk doordat ze relevant zijn in vele toepassingen uiteenlopend van industriële processen zoals reinigen, schuim flotatie en afvalwater behandeling tot in het biomedische veld als contrast middel voor ultrasoon geluid, medicijnen afgifte en diagnose van ziektes. Echter, studies binnen deze toepassingen zijn niet volledig, hebben een hoge spreiding en zijn niet systematisch uitgevoerd. Dit proefschrift poogt systematisch studies te verrichten naar interacties tussen nanobubbels en verschillende soft matter systemen zoals diverse colloïdale suspensies, liposomen en virale capside-eiwitten. Onze studie presenteert ook een reeks experimentele resultaten en controles om het begrip van nanobubbeleigenschappen te verbeteren.

Het proefschrift begint met het geven van een beknopt literatuuroverzicht in Hoofdstuk 1 over bulk nanobubbels, zoals generatie van nanobubbels,

*The english-to-dutch translation was provided by Ab Nieuwenhuis. I thank Ab for his invaluable help in translating the summary into Dutch. I also thank Guy Mendes de Leon for a press check.

karacteriseringsmethoden, stabiliteitsmechanismen en potentiële toepassingen. Ondanks de belofte van praktische implicaties, proberen we eerst een brug te bouwen om interacties van nanobubbels met soft matter systematisch te onderzoeken.

Voor het onderzoek beschreven in Hoofdstuk 2, hebben we eerst gekeken naar de interactie van bulk nanobubbels met negatief geladen Au-nanodeeltjes. Het resultaat van interactie tussen Au nanodeeltjes en nanobubbels suggereert dat nanobubbels direct op het nanodeeltje groeien na een kern gevormd te hebben op het oppervlak. Dit wordt bevestigd door het feit dat de kleinere Au-nanodeeltjes geen interactie en grote nanodeeltjes een sterke interactie hebben met nanobubbels. Wanneer botsingen de drijvende kracht zijn voor de interacties, verwachten we dat juist het kleinste nanodeeltje de sterkste interactie heeft door zowel de verlaagde DLVO-energiebarrière als de verhoogde diffusiesnelheid.

Vervolgens hebben we de interactie met positief geladen amidinepolystyreen deeltjes met verschillende afmetingen bestudeerd zoals gerapporteerd in Hoofdstuk 3. Wanneer we de amidine nanodeeltjes mixen met de nanobubbel oplossing nemen we “reentrant condensation” waar voor alle afmetingen van de nanodeeltjes. Dit proces is direct te volgen door naar de veranderende grootte van de nanodeeltjes en de elektroforetische mobiliteit van het mengsel te kijken. De studie naar de relatie tussen de concentratie van niet geladen nanodeeltjes en het totale oppervlak van alle nanodeeltjes geeft ons een beter begrip van het nucleatiemechanisme dat ten grondslag ligt aan onze waarnemingen.

In Hoofdstuk 4 maken we de stap naar liposomen in bulkoplossingen om de fenomenologie van bulk nanobubbel interacties in een organisch systeem te onderzoeken. We gebruikten dynamische lichtverstrooiing om de hydrodynamische en elektroforetische eigenschappen te onderzoeken en fluorescentiemicroscopie om de structuurverandering van nanobubbel-liposoomcomplexen te visualiseren. Dezelfde “reentrant condensation” werd

waargenomen met kationische liposomen, wat het interactiemechanisme van gas nucleatie op de liposoomoppervlakken verder ondersteunt.

Tenslotte, eindigt het proefschrift met een studie naar bulk nanobubble-geïnduceerd zelfassemblage van virale capside-eiwitten gedemonteerd en gezuiverd van natuurlijk CCMV. In Hoofdstuk 5 behandelen we de succesvolle zelfassemblage van capside-eiwitten met behulp van drie verschillende technieken, dynamische lichtverstrooiing, atoomkrachtmicroscopie en transmissie-elektronenmicroscopie. In dit proces functioneren bulk nanobubbels als sjablonen. We hebben ook vastgesteld dat afhankelijk van de eiwit concentraties, dezelfde bouwstenen capsides van verschillende groottes kunnen vormen, wat overeenkomt met verschillende bouwstructuren.

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