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Gold Nanoparticles as Injectable and Minimally Invasive Markers for
Real-Time Image Guided Radiation Therapy

画像誘導放射線治療用の注射可能な低侵襲金ナノ粒子マーカー



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ABSTRACT

Radiation therapy (RT) is one of the most common types of cancer treatments, and more than half of cancer patients receive RT as part of their treatment plan. However, the therapeutic effect of RT is governed by factors such as radioresistance and potential damage to surrounding tissues. Metal-based materials have the potential to tackle these limitations by acting in different roles, including radiosensitizers, imaging agents, delivery vehicles, oxygen generators, and radioprotectors. For example, gold-based fiducial markers are essential in real-time image-guided radiation therapy (IGRT). By identifying the fiducial marker, IGRT can accurately track the position of a tumor to avoid geometric uncertainties caused by body motions during RT. IGRT can therefore improve the precision of radiation delivery and reduce the doses to the surrounding tissues. However, currently used fiducial markers with large physical dimensions would lead to complicated and invasive insertion procedures, while injectable fiducial markers based on smaller-sized gold nanoparticles (Au NPs) are obviously promising and preferred in IGRT to relieve patients' pain and improve their clinical experience. Therefore, this study aims to synthesize Au NPs via green, effective, and biocompatible methods for fiducial marker applications to achieve optimal therapeutic efficacy and minimal treatment toxicity.

Chapter 1 is a general introduction of metal nanomaterials engineered for RT, especially the design considerations for metal nanomaterials and their applications for RT enhancements. The motivations and objectives of this study are also introduced.

Chapter 2 presents a one-step synthesis of sodium alginate-stabilized Au NPs (alg-Au NPs) using the microwave-induced plasma-in-liquid process (MWPLP). Effects of sodium alginate with various concentrations on the synthesis and properties of the synthesized Au NPs, including reaction rate, morphology, size, and optical absorption property, were studied. The introduction of alginate (1) accelerated the reaction rate, (2) prevented aggregation and deposition due to long-time discharge in

MWPLP, and (3) provided long-term colloidal stability. An abnormal size change (from large to small) of Au NPs during particle growth, which was opposite to the typical change in bottom-up chemical reduction, was observed and a possible mechanism was proposed based on the dynamical and thermodynamical instability of particles during growth. The strategy of drying and redispersion of alg-Au NPs in alginate solution was also studied. The drying and redispersion process had an imperceptible effect on the Au NPs. Consequently, this strategy might be an effective technique for the long-term storage of Au NPs. Alg-Au NPs without the addition of toxic reducing or stabilizing agents can be appropriate for biomedical applications.

Chapter 3 reports the synthesis of Au NPs via a green ethanol reduction method for the development of injectable fiducial markers. The factors that affect their injectability and imaging capability were also determined. Au NPs synthesized in the ethanol-only system had an improved injectability (through 18G and 21G needles) and their imaging performance was almost identical to current fiducial markers used in the clinic. The reducing capacity of the ethanol system was significantly enhanced through the introduction of sodium alginate. Meanwhile, the addition of sodium alginate could also control the size of Au NPs, ranging from 21.8 to 14.1 nm. The product alg-Au NPs with smaller sizes enabled an injectability up to 100% even through 25G needles with an extrusion force less than 17 N, which is superior for the injection of high-concentration Au NPs, exhibiting a minimally invasive property. The X-ray visualization test also verified a better imaging performance of alg-Au NPs than clinically used markers.

Chapter 4 describes a ready-to-use injectable fiducial marker based on Au NPs and a body temperature-activated *in situ* gel-forming system. Gram-scale alg-Au NPs were synthesized through MWPLP, and the body temperature-activated *in situ* gel-forming system was developed using sodium alginate and a temperature-sensitive calcium source (glucono delta lactone (GDL) and CaCO_3). The injectable fiducial marker GDL/ CaCO_3 /alg-Au NPs could remain liquid state at a low temperature to maintain its injectability. At body temperature, however, Ca^{2+} would be released as a result of high

temperature-activated hydrolysis of GDL and its subsequent reaction with CaCO_3 , initiating the gelation process of sodium alginate. The injectable fiducial marker can be delivered via injection and then gelate at the target site to inhibit marker movement or the leakage of Au NPs *in vivo*. Rheological characterization demonstrated the stability and gelation behavior of GDL/ CaCO_3 /alg-Au NPs at different temperatures. Furthermore, the injectability and imaging ability of GDL/ CaCO_3 /alg-Au NPs were also investigated.

Chapter 5 reviews the research findings and conclusions. The contributions and perspectives are also summarized in this chapter.

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Chapter 1. General Introduction

1.1. Radiation Therapy

Cancer is a primary cause of death worldwide, accounting for 10.0 million deaths in 2020.[1] It is a generic term for a vast group of diseases characterized by the unregulated growth of abnormal cells and the potential for invading adjacent organs or tissues. Currently available cancer treatment options include radiation therapy, chemotherapy, surgery, and immunotherapy. Notably, radiation therapy (also known as radiotherapy, RT) has become an integral part of cancer treatment due to the technological advances and improved understanding of its effects on the body since the discovery of radiation and radioactivity. More than half of cancer patients will receive RT alone or in combination with other therapies[2]. During RT, high-precision radiation is delivered to the tumor to induce the death of tumor cells. Radiation is carried by waves (gamma[3] and X-rays[4]) or a stream of particles (alpha,[5] beta,[6] proton,[7, 8] and neutron particles[9]). Cellular components and water will be ionized due to the high-energy radiation. In addition to charged species, radiation-mediated lysis of water molecules will also generate free radicals such as hydrogen radical $H\bullet$, hydroxyl radical $OH\bullet$, superoxide O_2^- and charged water species such as H_2O^+ .[10] DNA is the primary target of ionizing radiations and the generated radicals. RT can damage genes and prevent the growth and proliferation of tumor cells by breaking their DNA inside the cells. Free radicals can also interact with cell membranes and then lead to the structural damage and the death of tumor cells. In these ways, RT is used to (a) cure or shrink early-stage cancer, (b) keep cancer from recurring in another area, or (c) relieve symptoms caused by advanced cancer.

Depending on the radiation source, location, and modality, RT can be categorized into two types: external beam radiation therapy (EBRT)[11, 12] and internal radiation therapy (IRT).[13, 14] EBRT is the most common type of RT. It delivers radiation from a machine outside the body, such as X-ray machine, Cobalt-60 machine, linear accelerator, proton beam machine, or neutron beam machine, to the target lesions. EBRT is capable of treating large areas of the body, and more than one area could

be treated simultaneously, for instance, the tumor and nearby lymph nodes.[15] Therefore, EBRT has been extensively incorporated into the treatment protocol for local solid tumors of the head and neck area, breast, lung, colon, or prostate.[16-21] IRT, on the other hand, can be utilized to treat metastatic tumors as well as local tumors since radioisotopes are implanted as close as possible to the tumor site or inserted into the tumor in the forms of pellets, seeds, ribbons, wires, needles, capsules, balloons, or tubes. Radioisotopes can also accumulate in the tumor by intravenous administration through carriers with targeting moieties.[22, 23] IRT is particularly effective in the treatment of cancers that need a high dose of radiation to a small area, such as cervix, uterus, vagina, rectum, eye, and certain head and neck cancers.[14, 24-26]

Despite the effectiveness of RT in preventing and controlling the growth of most types of cancer, a number of cancer patients will suffer from possible damage to surrounding normal tissues, recurrence, and metastasis during and after RT. An ideal radiation dose should be sufficient to kill tumor cells while limiting the injury to healthy tissue by only damaging, not killing, nearby normal cells. However, high-dose radiation is typically required during the treatment due to the variability of radiation absorption coefficient between tissues, attenuation in the radiation direction, and tumor hypoxia-associated radioresistance.[27] Moreover, a radiation boost is essential to prevent cancer recurrence and metastasis. The elevated dose will consequently cause cell death in adjacent tissues and raise the risk of side effects, such as mouth and gum sores, swallowing difficulty, nausea, shortness of breath, and rectal bleeding. Patients treated with IRT also have to face the risk of off-target effects and the accompanying side effects, including tenderness, tightness, redness, and bruising. Accordingly, considerable attention has been given to the strategies to balance therapeutic advantages and physiological disadvantages.

Recent advances in nanotechnology have promoted the development of nanomedicines based on nanomaterials for enhancing radiation responses and overcoming limitations of RT, allowing an improved therapeutic outcome of cancer treatment.[28-30] The term nanomedicine is coined by the

National Institutes of Health (NIH) and refers to organic or inorganic nanomaterials for treatment, diagnosis, monitoring, or control of biological systems. In particular, metal nanomaterials have attracted significant attention owing to their small size, large surface area to volume ratio, strong X-ray mass attenuation coefficient, and their capabilities of passive or active targeting accumulation at tumor sites. Therefore, metal nanomaterials are promising as radiosensitizers,[30] imaging agents,[31] delivery vehicles,[32] radioprotectors,[33] or therapeutic agents to combine with other treatment modalities.[34] For example, high-Z metal nanomaterials with strong X-ray attenuation ability (e.g., gold, platinum, silver, gadolinium, hafnium, tantalum, tungsten, bismuth) can be used to enhance radiosensitization [35-38] and image contrast.[39-41] Interestingly, the tumor microenvironments (TMEs), which feature low pH, low-level of oxygen (hypoxia), high-level of glutathione (GSH) and reactive oxygen species (ROS), are different from those of normal tissue. Therefore, a growing number of studies have attempted to modify the surface of metal nanomaterials for active target delivery to tumor site based on the unique TMEs (low pH or hypoxia), or improving the limited therapeutic effect of RT arising from hypoxia and high-level GSH.[42-45] Moreover, metal nanomaterials RT enhancements can also be integrated with other treatment modalities, which will realize synergistic combination therapies for improved anticancer outcomes. While nanomedicines based on metal nanomaterials have been proposed to enhance RT efficacy for decades, tremendous advances and progress have been made recently. The objective of this chapter is to review the progress in the constructions of metal nanomaterial-based systems and their roles in RT enhancements. The primary focuses here are the design considerations for metal nanomaterials in terms of size, shape, surface modification and functionalization, administration route, as well as their applications as radiosensitizers, imaging agents, delivery vehicles, oxygen carriers or generators, radioprotectors, and therapeutic agents for RT enhancement (**Figure 1.1**).

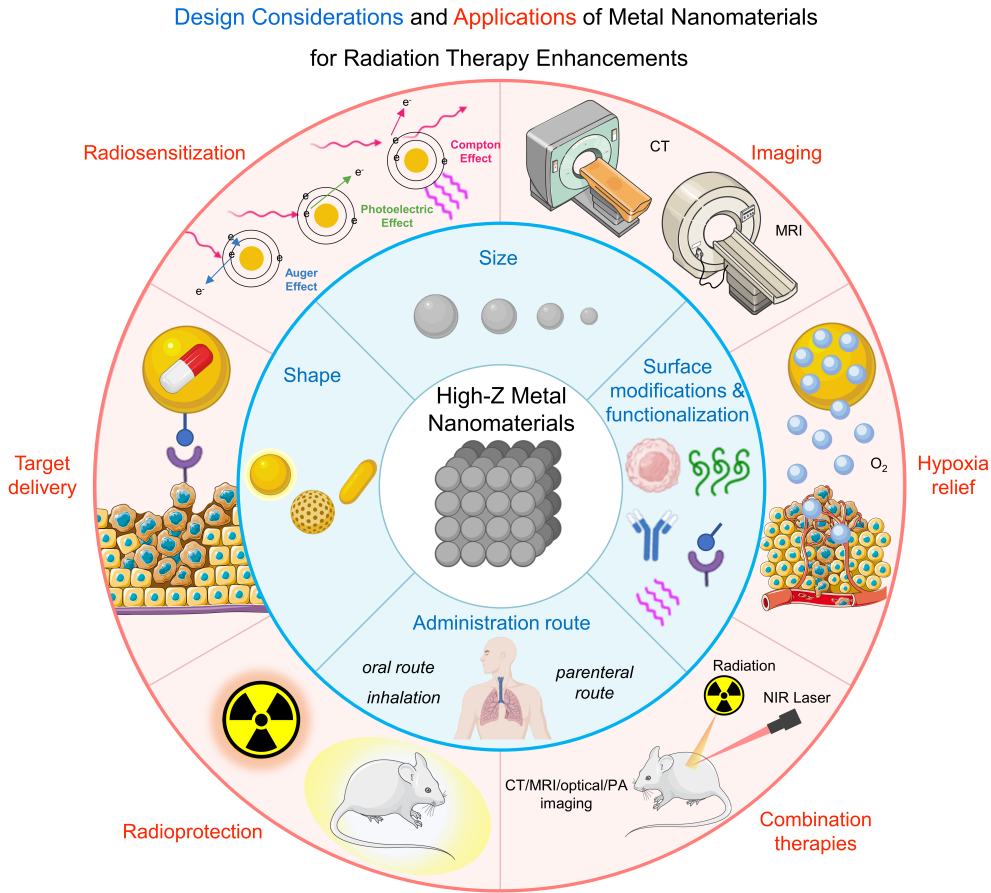


Figure 1.1. Schematic diagram of the design considerations and applications of metal nanomaterials for RT enhancements.

1.2. Design Considerations for Metal Nanomaterials for Radiation Therapy

The inherent properties of metal nanomaterials (e.g., high density, high attenuation coefficient, strong surface plasmon resonances (SPR)) ensure their promising applications in RT enhancements. However, given the environments of their usage scenarios and their specific functions at the target sites, several factors have to be taken into consideration: (a) uptake by cells, clearance by the kidney, liver, and the spleen, (b) potential toxicity, (c) biocompatibility during vascular circulation and after accumulation, (d) circulation time, (e) stability and biodegradability *in vivo*, (f) biodistribution, (g) function enhancements, such as target delivery, stimuli responsiveness, barrier penetration. In general, these properties and functions are dependent on the design of metal nanomaterials, primarily through

the modulations of their sizes and shapes, surface modifications and functionalization, and administration routes (**Figure 1.2**). The following are the overview of the structure designs, the strategies for their modification and functionalization, and the administration routes of engineered metal nanomaterials for RT enhancements.

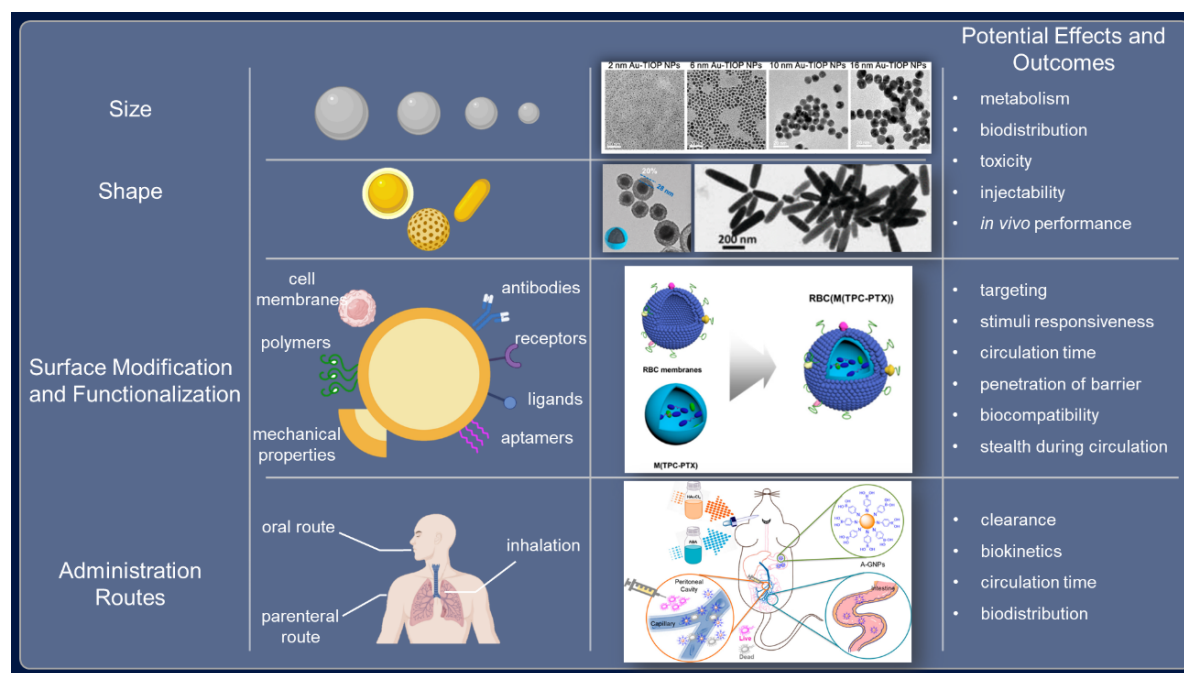


Figure 1.2. Design considerations and their potential effects and outcomes on metal nanomaterials for RT enhancements.

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1.2.1. Size Control of Metal Nanomaterials and Their Size-Dependent *in vivo* Behaviors and Performance

Size Control Strategies

There are two primary synthesis routes for the fabrication of metal nanomaterials: top-down and bottom-up. The top-down approaches involve synthesizing materials with desired sizes by etching

bulk materials (e.g., foils or pellets). As a representative top-down approach, laser ablation has inherent disadvantages and limitations, including low yield and difficult control over the shape and size of the synthesized nanomaterials.[51] Conversely, size control can be achieved more easily in bottom-up approaches, particularly chemical methods. For example, the Turkevich method is a classic and relatively simple technique for the synthesis of gold nanoparticles (NPs) between 10 nm to 30 nm. This method was then adapted by Frens et al. to synthesize gold NPs with an average size up to 150 nm by decreasing the amount of reducing agent.[52] The ratio of gold salt to reducing agent was verified to be the critical factor in controlling the size of gold NPs. They also proposed that a decreased amount of reducing agent can slowly reduce the gold ions but would not cap the particles and prevent further growth of the formed gold NPs. In contrast, by improving the reduction speed with a more potent reducing agent (e.g., sodium borohydride), the rapid reduction from gold ions to gold NPs can yield smaller and less polydisperse particles.[53] Other attempts have also been made to manipulate the size of metal nanomaterials by adapting the reaction conditions, such as pH,[54] temperature,[55] metal ion concentration,[56] stabilizing agent,[57] and solvent.[58] The core of these strategies is the regulation of the nucleation process and subsequent growth by altering the reaction conditions. For example, Li et al. investigated the effect of pH on the particle size and found that higher pH led to the formation of smaller silver NPs.[59] At the high pH range, the stability and colloid formation of clusters were higher due to the complete charging of the clusters. The formation process of NP at different pH values varied in the beginning nucleation/coagulation step or the final NP growth step. When the pH elevated, the accelerated reaction rate promoted the crystallization into smaller particles, which involved the nucleation and growth processes of smaller particles from Ag(0) nuclei, leading to an increase in the number of NPs and a decrease in their particle size.

Size-Dependent *in vivo* Behaviors and Performance of Metal Nanomaterials

Metabolism. The size of metal nanomaterials has a crucial role in their metabolism after entering the bloodstream through inhalation, ingestion, injection, or dermal exposure. Nanomaterials in the

bloodstream can be uptaken by vascular endothelial cells or extravasate into peripheral organs via transcytosis or paracellular pathways, and they would be cleared from the body in several ways. Renal clearance is the most effective excretion route for nanomaterials. The glomerulus in the kidney is a network of small blood vessels (capillaries), and it consists of a sandwich structure: capillaries, glomerular basement membrane (GBM), and podocytes. Capillaries are lined by endothelial cells with numerous pores, also known as fenestrae. These fenestrae are 50-100 nm in diameter, and the slit width of the slit diaphragms between podocytes is approximately 30-40 nm. Due to the integrated effect of the layers of the glomerular capillary wall, the functional or physiological pore sizes are about 4-5 nm.[60] Such structure renders the glomerulus highly size-selective, with only nanomaterials smaller than the renal filtration threshold (approximately 6 nm) can be cleared through kidneys, whereas the larger ones would retain in blood circulation.[60, 61]

Hepatic clearance is the major excretion route for the nanomaterials that cannot be cleared via the kidneys. The liver consists of parenchymal cells (hepatocytes) and nonparenchymal cells (Kupffer cells, liver sinusoidal endothelial cells (LSECs), stellate cells, and other cell types). Hepatocytes and Kupffer cells are two primary cells involved in the clearance of nanomaterials from the liver. After passing through the endothelium into the Disse space, nanomaterials with a diameter of 10-20 nm would be captured by hepatocytes and excreted via the bile into the small intestine following endocytosis and enzymatic breakdown.[60, 62] Due to the sufficient time for phagocytosis and micropinocytosis enabled by the slow blood flow in the liver (reduced 1000-fold, from 10-100 cm/s to 200-800 $\mu\text{m/s}$), nanomaterials larger than 200 nm will be cleared by the Kupffer cells, which are specialized macrophages in the liver and a part of mononuclear phagocyte system (MPS).[63] In addition to Kupffer cells, other macrophages accumulated in lymph nodes, the spleen (sinus histiocytes), and lungs (alveolar macrophages) would also contribute to the clearance of large nanomaterials.

In brief, small nanomaterials might be eliminated through renal clearance (< 6 nm) or rapid hepatic uptake (10-20 nm), while large nanomaterials (> 200 nm) will be cleared by the liver and spleen or captured and removed by MPS[61] (**Figure 1.3**).[64] Rapid clearance is a significant challenge for nanomaterials in biomedical applications. For a prolonged blood circulation time and specific accumulation in the target organs or tissues, size control is crucial in the design of metal nanomaterials for diagnostic or therapeutic purposes. Nanomaterials with diameters of 20-200 nm are considered to possess an extended blood circulation time since they are large enough to escape renal clearance and rapid hepatic uptake and small enough to prevent taking up by the spleen and the Kupffer cells in the liver.

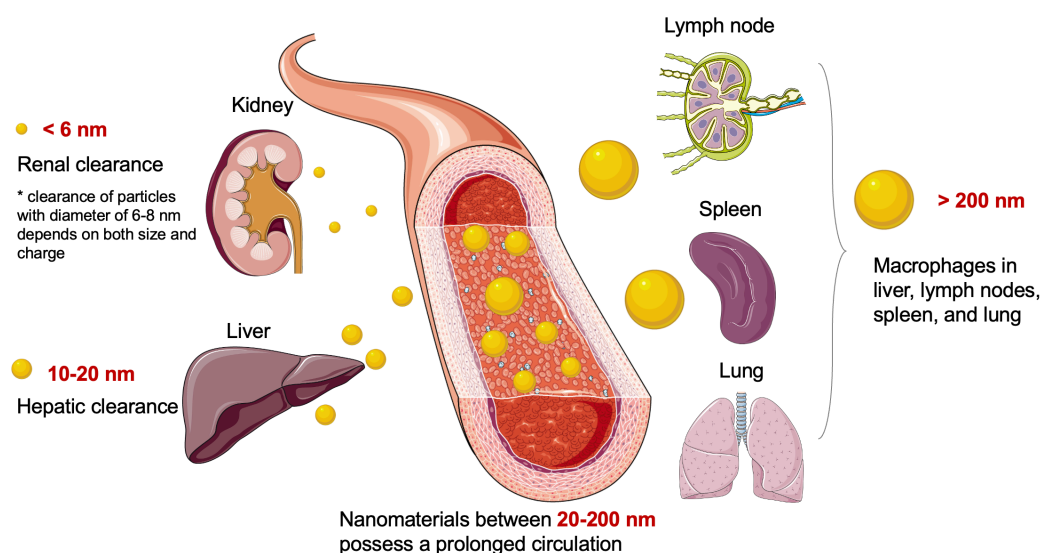


Figure 1.3. Clearance pathways of nanoparticles with different sizes after intravenous administration.

Biodistribution. The size of nanomaterials is also known to influence their biodistributions, which would further affect their accumulation concentrations at the target site and the determination of the dose required for the minimum effective concentration (MEC). Sonavane et al. investigated the effect of particle size on the biodistribution of colloidal gold NPs with particle sizes of 15, 50, 100, and 200 nm.[65] After 24 hours, the liver had the largest concentration of 15-nm gold NPs, followed by the lung, kidney, brain, and spleen. A larger concentration of gold was found in the liver, lung, and

spleen for 50- and 100-nm gold NPs. When the particle size was raised to 200 nm, the maximum gold concentration was detected in the liver, similar to the gold NPs of other sizes. Except for the liver, a higher concentration of 200-nm gold NPs was observed in the spleen than in the lung. Notably, considerable gold NPs with particle sizes of 15 and 50 nm were detected in the brain, suggesting gold NPs may pass across the blood-brain barrier (BBB). The biodistribution of nanomaterials with smaller sizes was studied in another work.[66] Compared to the gold NPs with sizes ranging from 5 to 200 nm, the concentration of ultrasmall gold NPs (1.4 nm) was the lowest in each tissue owing to the rapid clearance from the circulation through renal clearance.

Leaky vasculature coupled with poor lymphatic drainage during tumor formation allows nanomaterials to extravasate from the tumor vasculature and retain within the tumor, and this phenomenon is known as the enhanced permeability and retention (EPR) effect.[67] Since metal nanomaterials are designed to function at the tumor sites, the effect of size on their permeability and retention within tumors should be considered as well. Perrault et al. systematically examined the effect of NPs size (10-100 nm) on passive targeting of tumors *in vivo*. [68] The permeation of NPs within the tumor was highly size-dependent, where 20-nm particles could rapidly diffuse through the tumor matrix and migrate far into the interstitial space, while 60-nm particles showed limited migration and 100-nm particles would stay near the vasculature. This work highlights the essential role of size in the specific tumor targeting behaviors for different applications. For example, if the purpose is to maximize the diagnostic sensitivity by delivering the most metal nanomaterials into the tumor, nanomaterials with a diameter of 60-100 nm would provide the best performance.

Toxicity. It is of great importance to investigate the toxicity of metal nanomaterials since their applications *in vivo* depend on their circulation in the blood, as well as their accumulation in tissues and organs. Additionally, the size of nanomaterials determines how they interact with biological systems, thus it is vital to understand the effect of the size on their toxicity. Chithrani et al. conducted a study using gold NPs with an extensive size range (14, 30, 50, 74, and 100 nm) to quantitatively and

qualitatively evaluate the cellular uptake of NPs with different sizes.[69] The cellular uptake was found to be greatly dependent upon the size, and the maximum uptake by a cell occurred at a particle size of 50 nm. Further cellular viability assessment did not indicate any cellular toxicity due to the uptake of gold NPs (98% viability), which is consistent with the findings from other research groups.[70, 71] Gold NPs with sizes between 14 and 100 nm were observed to be trapped in vesicles in the cytoplasm, suggesting that gold NPs did not enter the nucleus, which may account for their lack of toxicity. Similarly, Jiang et al. demonstrated that the size of gold and silver NPs could influence membrane receptor internalization.[64] Notably, 40- and 50-nm gold NPs exhibited the greatest internalization, in agreement with the conclusions of Chithrani et al. Despite the low toxicity, the size of nanomaterials would still cause some changes in the biological system. For example, naked gold NPs ranging from 3 to 100 nm were injected intraperitoneally into mice to assess their potential impact on the biological system.[72] Although gold NPs with diameters of 3, 5, 50, and 100 nm had no adverse effects on mice, the gold NPs between 8 and 37 nm caused severe nausea. Mice injected with gold NPs in this range also suffered from fatigue, loss of appetite, fur changes, and weight loss. The mice had a camel-like back and crooked spine after two weeks. According to the pathological examination of major organs of the mice in the diseased groups, there was a remarkable increase in Kupffer cells, suggesting a large-scale immune response in relevant sites caused by the administrated gold NPs.

Injectability. Delivery of metal nanomaterials via injection is one of the most accessible options for clinic treatments. High-efficiency delivery of metal nanomaterials through injection can transfer an accurate and ideal dose to target organs or tissues to improve the therapeutic effect. A challenge to injection efficacy is the clogging or retainment of metal nanomaterials in the syringe during or after the injection, which might influence the therapeutic efficacy and clinical translation of the designed nanomaterials. Sarmadi et al. developed a multiphysics model, coupling computational fluid dynamics with microparticle transport to numerically analyze microparticle injectability in a syringe-needle

system.[73] The developed model evaluated the impact of multiple design variables, such as size, shape, concentration, initial distribution, needle gauge, and injection fluid viscosity, on microparticle injectability (Figure 1.4). The size has been shown to play a significant role in determining injectability rather than the concentration or shape, and a high concentration of small particles would pose a lower risk of needle blockage than a low concentration of large particles. This work highlights the effect of particle size on injectability and suggests that comparable injectability accompanied with programmable release kinetics can be achieved by changing particle morphology but retaining particle size.

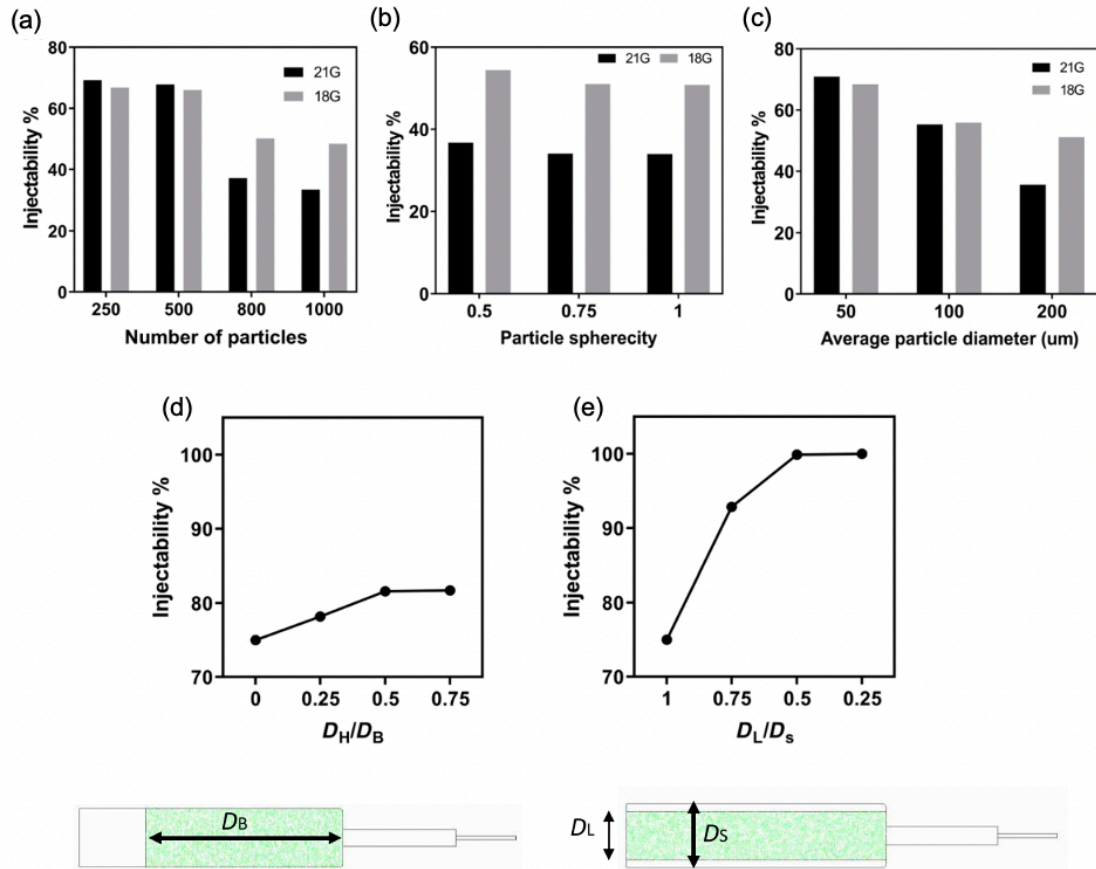


Figure 1.4. Numerical results demonstrated the effect of design parameters important in drug delivery on microparticle injectability. (a) The effect of particle concentration (200 μm in diameter, spherical) on microparticle injectability. (b) Effect of particle sphericity (shape) on injectability. (c) Injectability of microspheres (1000 particles) as a function of particle size. Effect of initial particle offset (10,000

particles) from (d) the plunger and (e) the syringe inner wall on injectability. Particles are illustrated as green dots in the syringe.

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In vivo Performance. A pioneering work found that tumors loaded with X-ray-absorbing metal microsphere at 1% by weight suffered 30-50% increased radiation damage.[74] The addition of metals could increase X-ray absorption and release more electrons, which will generate more ROS. In 2007, the evidence of molecular scale radioenhancement was first reported.[75] Low-energy electrons emitted from nanomaterials with X-ray radiation in an aqueous solution can deposit energy with nanoscale precision. A Monte Carlo method was developed to elucidate the energy deposition mechanisms by investigating the generation and transport of electrons in nanomaterials, in which small gold NPs were conjugated to supercoiled DNA. Single-strand breaks increased in the presence of gold NPs, resulting from the combination of three effects: (a) localized absorption of X-rays by nanostructures, (b) effective release of low-energy electrons from small nanostructures, and (c) efficient deposition of energy in water in the form of radicals and electrons. Zhang et al. further addressed the size-dependent enhancement of radiation effects of gold NPs.[76] All sizes of polyethylene glycol (PEG)-coated gold NPs dramatically decreased cancer cell survival after gamma radiation, with 12.1- and 27.3-nm PEG-coated gold NPs having a dispersed distribution and higher sensitivity than 4.8- and 46.6-nm particles through cell necrosis and apoptosis. Furthermore, all sizes of PEG-coated gold NPs can decrease the tumor volume and weight after 5 Gy radiations. Likewise, 12.1- and 27.3-nm PEG-coated gold NPs had superior sensitization effects than 4.8 and 46.6-nm particles, which resulted in nearly complete tumor disappearance. Another work also supports the size-dependent radiosensitization of metal nanomaterials, in which the highest radiosensitization efficacy was observed for the 26-nm gold NPs size under 200 kVp voltage compared to gold NPs with other sizes (12, 15, or 21 nm).[77] Notably, the continued increase in size would negatively impact

the radiosensitization efficacy by increasing the internal absorption of secondary radiation and reducing the yield of superoxide radicals.[78, 79]

Moreover, other *in vivo* performance, e.g., imaging performance, will also be affected by the size of metal nanomaterials. Xu et al. studied the size effect of gold NPs on the attenuation to X-rays used for computed tomography (CT).[80] Smaller gold NPs were found to have a higher X-ray attenuation compared with larger ones at the same gold concentration. In another work, the increase in X-ray attenuation was also observed when the core sizes of the gold NPs decreased from 12 to 6.6 nm.[81] With the same gold concentration, smaller gold NPs could offer a larger gold surface area. Since the X-ray attenuation is target area-dependent, small gold NPs with a larger surface area would exhibit more dramatic X-ray attenuation. Meanwhile, the smaller size implied that a large number of gold NPs would distribute in the path of X-ray transmission, which increased the absorption or deflection (scatter) of photons and eventually led to X-ray attenuation as well.[82]

1.2.2. Shape Control of Metal Nanomaterials and Their Shape-Dependent *in vivo* Behaviors and Performance

In terms of shape, recent research has suggested that the shape of nanomaterials also has a significant impact on their *in vivo* behaviors and performance.[83-85] By fine-tuning and controlling the shape of the metal nanomaterials, a wide range of applications can be accessed. For example, gold nanorods (NRs) exhibit two SPR bands, where the one at 520 nm is attributed to transverse oscillations or surface plasmons oscillating along the short edge of gold NR. Surface plasmons oscillating along the long edge of the rod would produce a strong longitudinal plasmon band. The position of the longitudinal band is determined by the aspect ratio of gold NRs and is typically found in the near-infrared (NIR) range at and above 750 nm. In biological tissue, wavelengths over 750 nm are minimally scattered and absorbed, implying that imaging live targets with gold NRs is feasible due to the ability of NIR light to penetrate deep into the skin without interference from tissues. Controlled growth of isotropic or anisotropic structures can come from complex physicochemical

phenomena. The mechanisms behind the controlled growth have been investigated through the nucleation and growth processes, including adsorption growth, aggregation and agglomeration, oriented attachment, seeded growth, and self-assembly.

Adsorption Growth. Surface free energy and the number of surface irregularities, such as steps and kinks, greatly determine the growth rate of NPs, and particle surfaces having larger surface free energy and a greater number of kinks will grow faster to achieve equilibrium of NPs surface. Under thermodynamic control, sufficient energy supply in the reaction environment promotes the formation of isotropic (spherical) NPs instead of anisotropic ones. On the other hand, nanomaterials with desirable shapes could be synthesized by selectively attaching or growing onto specific facets of NPs using additives or surfactants to lower face tension and alter surface properties. For example, according to Saw et al., Cl^- ions significantly affect the shape of copper particles in an organic phase without capping agents.[86] Anisotropic copper rods could be produced with only Cl^- ions present. The rods had (011) facets as the long axis, (111) facets as the tip, and (100) facets as the side surface. More copper atoms contributed to the formation of copper rods when the Cl^- ion concentration was increased, indicating Cl^- ions preferentially adsorbed on (100) copper surfaces to promote the anisotropic growth of copper. In addition, at high Cl^- concentrations, the adsorption of Cl^- to the (111) and (100) surfaces would regulate the relative growth of the particle length and diameter. A plausible theory for selective bonding between additives (or surfactants) and the surface of NPs is that the electronic charge of a facet can adsorb or coordinate with the opposite charge from additives or surfactants. The specific adsorption can regulate the growth rate and the introduction of lattice distortions, which eventually determines the shape of synthesized nanomaterials.

Aggregation and Agglomeration. Since nanomaterials have high surface free energy, they tend to agglomerate and aggregate after collision during Brownian or flow motion to minimize the surface boundary between nanomaterials and solvents for a lower surface free energy. The aggregation has always been avoided in the colloid science process due to unpredictable morphology, undesired

features, and unsatisfactory applications. However, it has been shown that this phenomenon can be utilized to create nanomaterials with programmable morphologies. For example, nanomaterials with various anisotropic shapes, such as one-dimensional,[87] cubic, and octahedral,[88] can be synthesized by counterbalancing attractive forces with repulsive forces via the presence of capping agents or surfactants.

Oriented Attachment. Oriented attachment represents a growth mechanism for the self-organization of adjacent particles followed by the fusion among the formed NPs and the formation of a planar interface sharing crystallographic orientation. Oriented attachment allows the synthesis of different anisotropic nanostructures, such as NRs, wires,[89, 90] nanoplates,[91] and nanorices.[48]

Seeded Growth. Seeded growth is a versatile synthesis route to anisotropic nanomaterials through nucleation on the surface of pre-nucleated seeds. The structure, concentration, and growth kinetics could be independently regulated, allowing for convenient and accurate control of the size and shape of the target nanomaterials, which are typically in a core-shell structure.[47, 92]

Self-Assembly. Self-assembly is another synthesis approach of nanomaterials with a designed shape by mediating non-covalent or weak covalent interactions (electrostatic, van der Waals, hydrophobic interactions, hydrogen bond, and coordination bond). In contrast to oriented attachment, self-assembly is a controllable process involving the management of ligands or bonds among particles, while oriented attachment is less controllable as it highly depends on the reaction conditions and process.

With these mechanisms, various parameters, such as the concentration of the precursors,[93, 94] solvents,[95, 96] pH,[97, 98] additives,[99, 100] and surfactants,[101] can be regulated to control the shape of nanomaterials. The shapes of nanomaterials, like their sizes, can impact their circulation, uptake, metabolism, biodistribution, and other *in vivo* performance (**Figure 1.5**)[102-104]. Moreover, the shape of nanomaterials can be engineered to implement designed functions. For instance, Bi₂Se₃

hollow nanocubes (HNCs) were fabricated for low-temperature photothermal and RT combined therapy.[105] The unique shape endowed HNCs with a strong NIR region optical absorption and high tissue absorption coefficient. Consequently, HNCs had a higher *in vivo* photothermal conversion efficiency, which enabled photothermal therapy at a relatively low temperature (about 43 °C) and induced effective cancer cell apoptosis under mild NIR laser irradiation. Moreover, due to the shape design, HNCs with a larger free volume can carry more therapeutic agents than other reported Bi₂Se₃ NPs. The loading of gambogic acid, a type of heat-shock protein (HSP) inhibitor, into Bi₂Se₃ HNCs was capable of preventing cancer cells from being restored with the assistance of HSP. In another work, gold NRs and NPs with similar sizes were synthesized to evaluate shape effects on tumor accumulation and therapeutic efficacy.[104] According to the physiologically based pharmacokinetic model, gold NRs diffused more quickly in tumor interstitial than gold NPs. In addition to a higher distribution coefficient, gold NRs had a substantially higher tumor maximum uptake rate constant and a more effective inhibition of tumor growth than gold NPs, indicating the significant influence of shape on their tumor accumulation and chemoradiotherapy effect.

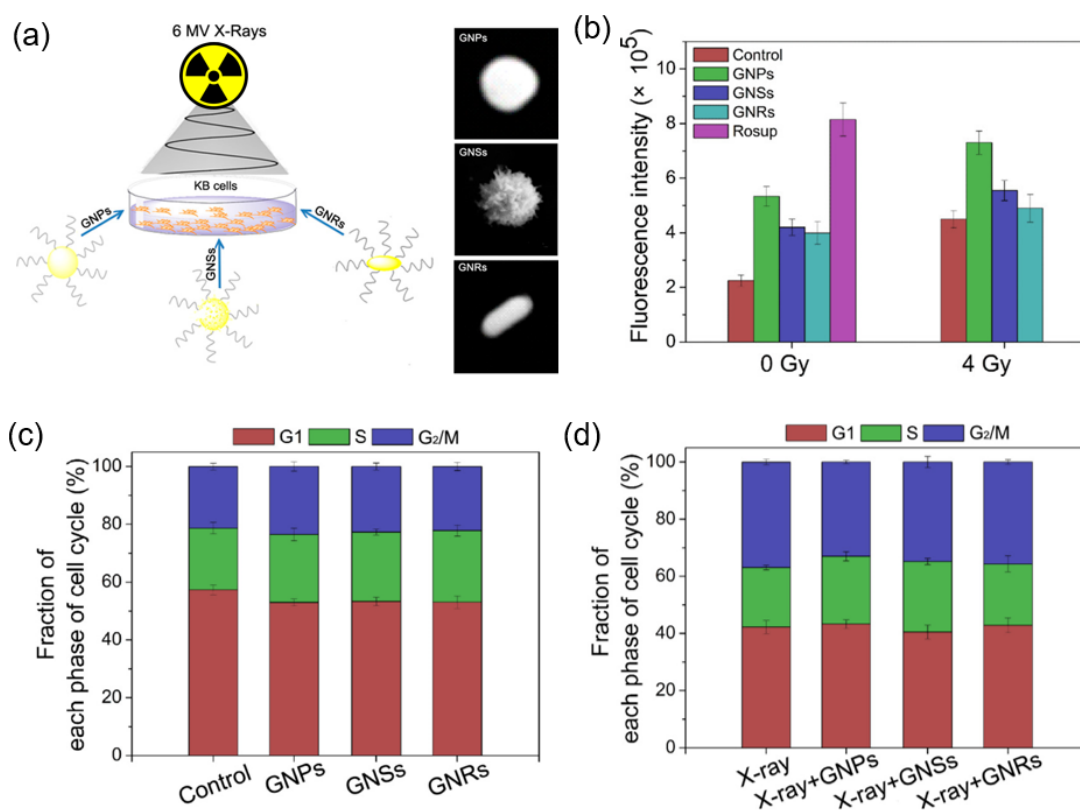


Figure 1.5. The shape-dependent radiosensitization of gold nanomaterials. (a) Schematics of the PEGylated gold NPs, gold NSs, and goldNRs. (b) Generation of ROS within human oral epidermoid carcinoma (KB) cells without (control) and with the treatment of gold NPs, gold NSs, and goldNRs under the X-ray irradiation of 0 or 4 Gy. Cell cycle distribution histograms of KB cells treated with gold nanostructures for 24 h without (c) or with (d) X-ray irradiation (4 Gy).

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1.2.3. Surface Modifications and Functionalization

To cope with the complex biological environments in the body while performing the designed functions of metal nanomaterials, surface modifications and functionalization of these nanomaterials are required to provide additional properties, e.g., active tumor targeting, stimuli responsiveness, performance enhancements (circulation time, penetration of barrier, biocompatibility, stealth during circulation), or altered mechanical properties.

1.2.3.1. Targeting

Nanomaterials can be delivered through passive targeting, e.g., through the EPR effect, but delivering nanomaterials more specifically to targets can significantly improve their therapeutic efficacy. By contrast, active targeting primarily relies on the biological characteristics of tumor cells or tumor tissue to increase the accumulation of nanomaterials within tumors. The biological characteristics usually refer to the receptors, ligands, or extracellular proteins expressed in high quantities on tumor cells or in tumor tissues.[106] For instance, during the rapid proliferation stage of a tumor, the transferrin receptor would be overexpressed. With its deep involvement in iron homeostasis and cell growth regulation and its vital role in extracellular access, internalization, and cellular pathology, transferrin receptor would be 100 times higher than the typical expression of normal cells.

Another well-known tumor marker is the folate receptor. In biological systems, folic acid is a necessary raw element for the production of purines and pyrimidines. These receptors are detected at significant levels on the membranes of tumor cells. It will come in two forms, folate receptor α and β , and folate receptor α is found overexpressed in 40% of human tumors.[107]

Moreover, glycoproteins and human epidermal growth factor receptor (HER), which are highly expressed in ovarian, kidney, pancreatic, and breast cancer, are also essential targets for active targeting.[108, 109]

The rate of blood supply accessible to solid tumors determines their development and proliferation, indicating that angiogenesis-related growth factors and their receptors will be expressed during this stage. The vascular endothelial growth factors (VEGF) and their receptors, VEGFR-1 and VEGFR-2, have been reported to be inducers of tumor angiogenesis and neovascularization. The high-level expression of VEGF promotes the proliferation and metastasis of endothelial cells as well as the construction of a matrix for neoangiogenesis.

Vascular cell adhesion molecule-1 (VCAM-1) is another transmembrane glycoprotein expressed on endothelial cells. VCAM-1 promotes adhesion between cells and is overexpressed in leukemia, nephroblastoma, melanoma, renal cell carcinoma, lung, breast, and gastric cancer.[110] The matrix metalloproteins (MMPs) can remodel the extracellular matrix (ECM), and they are pivotal in angiogenesis and metastasis by mediating the invasion and migration of endothelial cells. During the tumor growth, tumor cells will secrete proangiogenic chemokines, which further lead to an imbalance in the TMEs and influence the angiogenic switch. The VEGF and fibroblast growth factor (FGF) receptors can promote angiogenesis by remodeling ECM with the assistance of MMPs and the proliferation of endothelial cells.

The incorporation of integrins, such as $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$, is fundamental to this process. Therefore, integrins can also serve as targets for active targeting.[111] Compared to active targeting by tracking markers associated with the rapid proliferation of tumor cells, the main advantage of angiogenesis-mediated active targeting is that these markers are highly expressed regardless of the tumor type, which extends their application spectra in active targeting.

Nanomaterials can be decorated with targeting moieties that bind specifically to these receptors, ligands, or proteins on the tumor cell surface or in tumor tissue to improve their conjugation with the tumor. For example, antibodies can be used to recognize specific proteins or receptors on cancer cell membranes and some monoclonal antibodies have been approved by U.S. FDA for clinical use, such as Rituximab (Rituxan®), Alemtuzumab (Campath®), Avelumab (Bavencio®), and Ramucirumab (Cyramza®). Chen et al. developed a bimodal dendrimer conjugate selective for HER-2 to improve CT and magnetic resonance imaging (MRI) of HER-2-positive breast cancer.[112] As a humanized monoclonal anti-HER-2 antibody, trastuzumab is the mainstay in targeted therapy for HER-2-positive breast cancer. Trastuzumab was used with a generation 5 PAMAM dendrimer, gold NPs, and DOTA-gadolinium for subsequent CT and MRI imaging of breast cancer tumors *in vivo*. There was a 13% increase in signal intensity on CT scans 4 h after conjugate administration with the presence of

trastuzumab. Like CT, MRI demonstrated that signal intensity was significantly enhanced by 30% with trastuzumab compared to the non-targeted imaging agent, suggesting the antibody (trastuzumab)-guided dual-imaging conjugate can precisely target HER-2 positive breast cancers and improve both MRI signal and CT resolution.

Due to the capability of binding to a wide range of targets (e.g., intracellular proteins, transmembrane proteins, soluble proteins) via the interactions between nucleic acid and ligand, aptamers (Apts) can also serve as targeting agents.[113] Apts are short single-stranded DNA or RNA formed from a large random sequence pool.[114] Compared to antibodies, aptamers with smaller sizes possess high affinity and specificity, low immunogenicity, and better stability in different pH, temperature, and solvent conditions.[115] Recently, a tumor cell-specific “switch-on” photodynamic therapy nanoplatfrom (gold NR/Apt-Ce6) was established by conjugating gold NR and Chlorin e6 (Ce6) using a well-designed hairpin structure muc1 protein (MUC1) aptamer specific linker (**Figure 1.6**).[116] This system can switch on by conformational changes in the apt-induced fluorescence resonance energy transfer missing between gold NR and Ce6 in order to overcome the phototoxicity risk associated with photodynamic therapy. In the absence of tumor cells, MUC1 Apt maintained a hairpin structure, which resulted in Ce6 being tightly bound to the surface of gold NR, while photodynamic therapy remained in an off state, although nanoplatfroms were exposed to irradiation. On the other hand, when MUC1 was overexpressed in the presence of tumor cells, Apt preferentially identified and attached to MUC1, leading to Apt conformational alterations from a typical hairpin to an extended chain shape. Thus, when the distance between Ce6 and gold NR was increased, photodynamic therapy was activated. Due to its strong tumor-targeting and “switch-on” property, gold NR/Apt-Ce6 demonstrated reduced phototoxicity and remarkable photodynamic therapy efficacy in tumor-bearing mice (55.1% vs. 1.3%, tumor apoptosis rate).

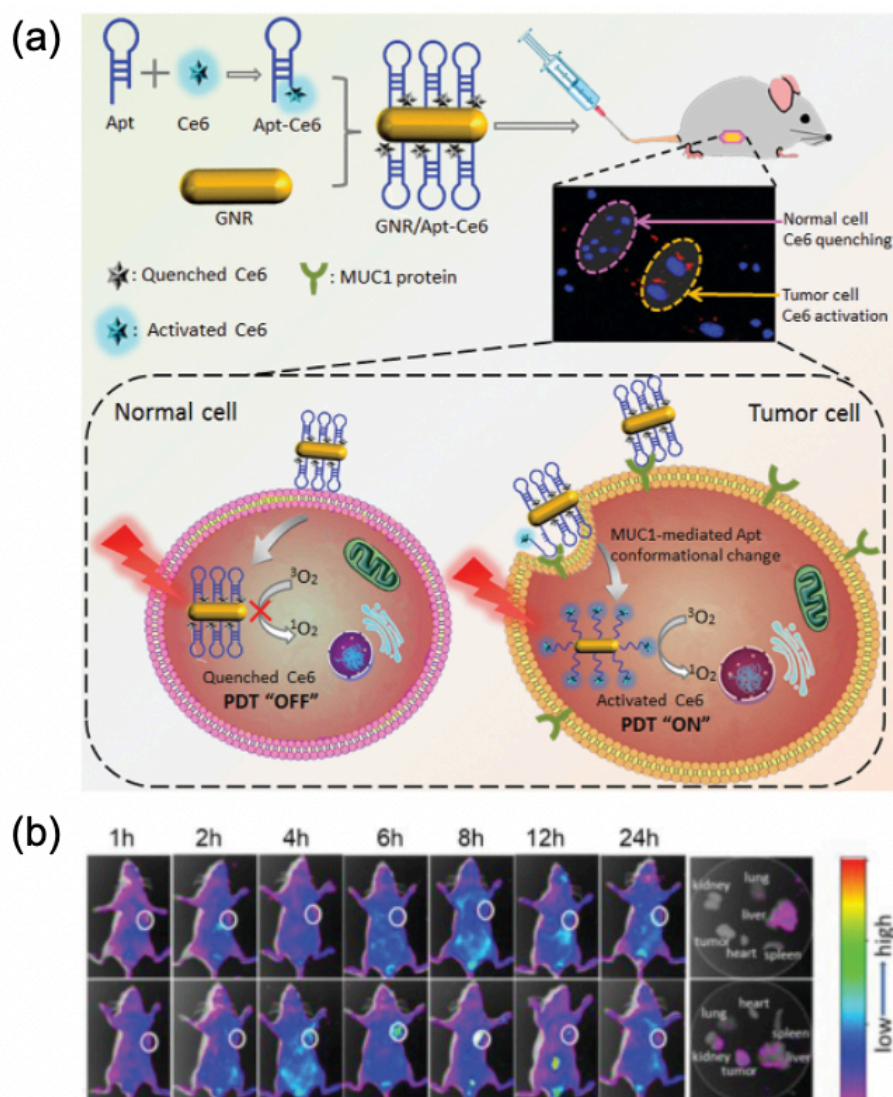


Figure 1.6. Target delivery using Apts. (a) Schematic illustration of the synthesis of gold NR/Apt-Ce6 and its MUC1-mediated “ON/OFF” PDT. (b) Target delivery and accumulation of gold NR/Apt-Ce6 within tumors.

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1.2.3.2. Stimuli Responsiveness

Endogenous Stimuli. As mentioned above, TMEs are distinct from that in normal tissues, but they can promote the aggressiveness, metastasis, and recurrence of tumors. TMEs are commonly regarded as a significant impediment to some delivery systems, but in turn, these characteristics of

TMEs can also turn into different types of stimuli and further act as triggers to control and increase the release of therapeutic agents in tumors. pH is one of the most frequently used characteristics to activate or control the release of payloads. Warburg observed that tumors consumed vast amounts of glucose in comparison to surrounding tissue, and glucose would be fermented to preferentially produce lactate even in the presence of oxygen, which is referred to as the “Warburg effect”.[117] The Warburg effect and subsequent lactic acid accumulation are directly responsible for the low pH in tumors, reaching as low as 6.5. Numerous delivery systems have been developed to transport or release their payloads by exploiting the minor pH difference between normal tissues (7.4) and tumors (6.5-7.2). Yang et al. used tamoxifen (TAM) to construct biocompatible nano-photosensitizers (nano-PSs) by triggering the self-assembly of Ce6-modified human serum albumin (HSA).[118] The formed HAS-Ce6/TAM nanocomplexes are stable at neutral pH but dissociate into individual HAS-Ce6 and TAM molecules in an acidic TME due to the pH-responsive transition of TAM from hydrophobic to hydrophilic. Accordingly, HAS-Ce6/TAM NPs exhibited dramatically improved penetration capability in tumors. In another work, pH also served as a trigger to dissociate dextran for the release of hyaluronidase, which would induce the breakdown of ECM for the subsequently enhanced penetration of oxygen and other therapeutic agents.[119]

Hypoxia is a direct consequence of hypervascularity in the tumor.[120] By lowering the diffusivity of solutes and fluid and increasing the oxygen demand, crowded tumor parenchymal cells contribute to the intratumoral hypoxia.[114] Many bioreductive anticancer medicines with high hypoxia-specific cytotoxicities, such as Tirapazamine (TPZ), AQ4N, and Mitomycin C (MMC), have been developed and used in the treatment of hypoxic malignancies as they can be activated in hypoxic environments.[121, 122] Apart from directly exploiting the properties of hypoxic tumors, another strategy is utilizing the unique feature for hypoxia-targeting. A size-tunable nanocluster bomb (33 nm) that can degrade to release tiny NPs (5 nm) was designed with hypoxia-targeting capability (**Figure 1.7**).[123] The nanoclusters bomb had a long half-life during blood circulation due to their enlarged

size. The destruction of the nanocluster bomb and the release of small NPs can be activated within tumors. These NPs can deeply penetrate into the tumor because of their smaller sizes and hypoxia-targeting capability. Therefore, high-spatial-resolution hypoxic profiles of solid tumors via enhanced CT and the validated therapeutic outcomes were enabled. In general, utilizing hypoxia as a target to overcome the hypoxia-induced restrictions and improve the therapeutic effect seems feasible and reliable.

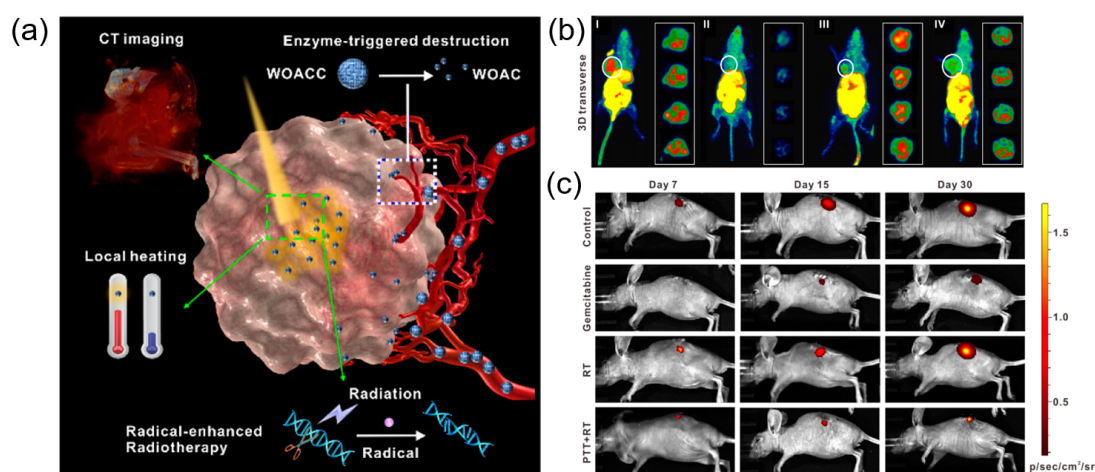


Figure 1.7. (a) Scheme illustration showing how MMP2-responsive WOACC NPs integrate active hypoxia-targeting properties with CT imaging, local PTT, and radical-enhanced RT. These WOACC NPs maintained their original size during blood circulation and destructed to release hypoxia microenvironment targeting WOAC NPs upon MMP2 simulation. (b) PET images of mice received different treatments. (c) Bioluminescence imaging of orthotopic pancreatic tumor-bearing nude mice following treatments with saline (Control), gemcitabine, RT, and synergistic treatment with WOACC NPs (WOACC).

Adapted with permission from [123]. Copyright 2017 American Chemical Society.

GSH, a γ -glutamyl-cysteinyl-glycine tripeptide, is an effective antioxidant that can protect cellular components from damage caused by ROS. The concentration of GSH in tumor cells is 100- to 1,000-fold higher than that in blood and other normal tissues.[124] The thiol groups of GSH allow it to work as a reducing agent, thereby developing a smart platform that can respond to GSH would be a

promising strategy for tumor-targeting treatment. For example, a NIR fluorescence-guided theranostics nanoplatfrom was constructed by integrating tumor response and photodynamic/photothermal therapy.[125] CyPT is a novel NIR fluorescent dye with excellent optical and photodynamic/photothermal therapy capabilities. CyPTs were attached to gold NRs through a sulfur-sulfur bond. GSH can break these sulfur-sulfur bonds with high selectivity and sensitivity. As a consequence, the fluorescence of CyPTs would stay quenched in normal cells with low GSH concentrations, while these sulfur-sulfur bonds will break due to the high concentration of GSH in tumor cells, inducing the release of CyPTs and the “off-on” fluorescence response. The photodynamic/photothermal therapy, which relied on the release of CyPTs and gold NRs, could be effectively performed following an accurate tumor-imaging diagnosis using NIR.

Exogenous Stimuli. Delivery systems based on exogenous stimuli (e.g., light, magnetic field, and ultrasound response) can provide many advantages over endogenous stimuli-based delivery systems, as they allow for more precise control of treatment timing and location. Many photo-responsive delivery systems can release carried therapeutic agents in response to UV or visible light irradiation. Due to the limited tissue penetration of UV or visible light, a number of studies have also used NIR light in the wavelength range of 650-900 nm as a light stimulus. For example, a NIR laser-responsive delivery system was synthesized for anticancer drug delivery.[126] The thermally reactive polymer showed a coil-to-globule volume phase transition in response to temperature change. When the system was subjected to a NIR laser, gold NRs absorbed light and emitted energy, followed by the aggregation of the polymer due to the generated heat and the release of the contained drugs for anticancer treatment. In addition to light-induced aggregation, light-triggered crosslinking of gold NPs was utilized for improved RT and CT imaging of tumors.[127] Gold NPs were decorated by conjugating folic acid and photolabile diazirine group on the surface of gold NPs. The crosslinking of gold NPs can be activated by 405-nm laser irradiation to enhance the accumulation and retention of

gold NPs within tumors, which further enabled remarkably improved tumor targeting ability and radiosensitization effect.

The magnetic field is another exogenous stimulus that shows excellent potential for manipulating *in vivo* behaviors of engineered nanomaterials. Functionalized nanomaterials can be directed to the tumor site using a magnetic field applied from outside the body, which allows a high-concentration accumulation of nanomaterials at the tumor site for a prolonged period while also protecting healthy tissue from potential side effects. A smart, magnetically targeted, TME-responsive nanocatalyst was constructed for cancer oxidation therapy via a specific reaction at the tumor site.[128] The core of these magnetic nanocatalysts contained a large number of enzymes, and their accumulation at the tumor site can be considerably improved by magnetic field-mediated orientation. In another work, the magnetic field was used to control the release of the loaded anticancer drug, in which mesoporous silica with magnetic cores was prepared as a carrier for doxorubicin (DOX) (**Figure 1.8**).[129] The presence of magnetic cores and gold NPs gave the system a sensitive response to the alternating magnetic field. The “on-off” magnetic field enabled a targeted delivery of DOX to the tumor and a more rapid accumulative release.

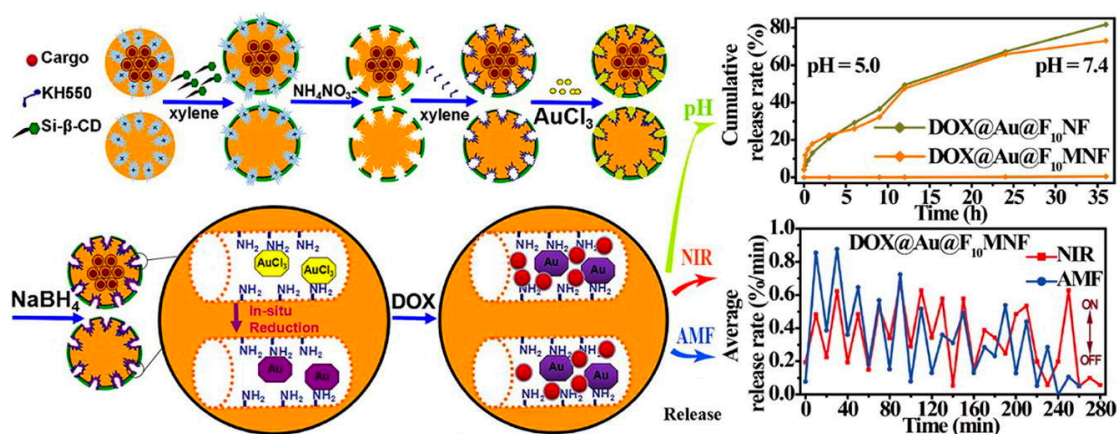


Figure 1.8. The synthesis process of the smart drug delivery nanosystems and its “on-off” responsive release in pH, NIR, and AMF conditions.

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Ultrasound is also an extensively used exogenous stimulus for its minor energy attenuation, high tissue-penetrating capability, accessibility, low cost, portability, as well as its ability to focus on the target with high precision in a non-invasive manner. Like the photo and magnetic response, ultrasound has a wide range of applications in controlled release and targeted delivery.[130-133] More interestingly, ultrasound was also employed as a microscopic pressure resource to generate ROS for piezocatalytic tumor therapy under catalytic mediation by piezoelectric tetragonal BaTiO₃. [134] Under the ultrasonic vibration, the electrons and holes were unpaired and separated by the piezoelectricity, a strong built-in electric field was established accordingly and then catalyzed the generation of ROS *in situ* for tumor eradication. This technique has a number of exciting advantages over conventional sonoluminescence-activated sonodynamic therapy, including more stable sensitizers and dynamic control of redox reaction outcomes.

1.2.3.3. Performance Enhancer

In general, surface modifiers will be introduced to enhance the *in vivo* performance of nanomaterials, such as stability, barrier-penetrating capability, and biocompatibility. For example, surface modifiers can provide nanomaterials with prolonged circulation time and stealth, which are essential for the adequate accumulation of nanomaterials at the target site. PEG is a typical surface modifier that can serve as a barrier to avoid the absorption of proteins and phagocytic processes. Since PEG is non-immunogenic and non-antigenic, a PEG-containing bisphosphonate anchoring group was utilized to impart hydrophilicity and antifouling characteristics to the synthesized particles.[135] The PEG formed a dense brush-like layer on the surface of the particles, which could increase their circulation time *in vivo* and prevent their aggregation in water and PBS. Over 70% of NPs still remained in the bloodstream after four hours, validating the prolonged circulation time and the capability to evade detection and degradation by the immune system.

The biological barriers can protect the body from the administrated substances and facilitate or impede the distribution and delivery of nanomaterials. Therefore, the barrier-penetrating capability is also necessary for nanomaterials to reach and accumulate at target sites. As a representative barrier, BBB is an interface that mediates the communication between the periphery and the central nervous system (CNS) and regulates the passage of solutes from the circulating blood to the CNS. Brain capillary endothelial cells (BCECs) that line the brain side of BBB confer the majority of the selective properties of the barrier. The tightly connected BCECs function as a high-resistance barrier to the transport of tiny hydrophilic molecules and ions.[136] Bare gold NPs and PEG modified-gold NPs were prepared to investigate the effect of surface modification on their biodistribution after intravenous administration.[137] The accumulation of gold NPs in the liver and spleen was significantly reduced by PEG, while the accumulations of PEG modified-gold NPs in the brain were 3.6 times and 2.7 times higher than those of bare gold NPs, demonstrating that surface modification can facilitate the passage of nanomaterial across the BBB.

Furthermore, surface modifications are also capable of improving biocompatibility and lowering the risk of cytotoxicity and side effects. In addition to natural and synthetic polymers, such as chitosan, alginate, PEG, and poly(lactic-co-glycolic acid) (PLGA), cell membranes, e.g., stem cell membrane, bacterial outer membrane, macrophage membrane, have emerged as an attractive ingredient for the modification of nanomaterials due to their ease of functionalization and superior biocompatibility.[138] Due to the “identity information” on the cell membrane, nanomaterials coated with cell membranes are considered as ordinary cells, and their biocompatibility is significantly improved. For instance, red blood cell (RBC) membranes were used to coat NPs for synergistic chemo/photodynamic therapy. The coating using RBC membranes can dramatically enhance the biocompatibility, blood circulation, and tumor accumulation of the engineered NPs.[49] More interestingly, surface modifications with cell membranes can confer diverse functions to nanomaterials as well, including specific recognition and immunotherapy.[139, 140]

1.2.3.4. Mechanical Properties

Systematical research on the effects of the size, shape, and surface modification and functionalization of metal nanomaterials on their properties, *in vivo* behaviors, and performance has been conducted. More recently, the mechanical properties of nanomaterials have also been demonstrated to regulate their biological behaviors and *in vivo* performance.[141] Uniformly sized PEG-based hydrogel NPs (200 nm) with elastic moduli ranging from 0.255 to 3000 kPa were synthesized to investigate the impact of particle elasticity on their *in vivo* behaviors such as blood circulation time, biodistribution.[142] Softer NPs (10 kPa, elastic modulus) had a better blood circulation performance and an enhanced targeting capability than harder NPs (3000 kPa). The softer NPs exhibited significantly reduced cellular uptake by immune cells, endothelial cells, and cancer cells. Since the majority of endogenous substances in the bloodstream, e.g., normal red blood cells, have low stiffness, whereas irregular or diseased cells and foreign substances have higher stiffness, the prolonged blood circulation of soft NPs may be attributed to the lower capture and clearance during circulation. Moreover, the accumulation of stiffer NPs in the spleen was remarkably higher than that of softer ones, indicating that soft NPs can deform and squeeze through the filtration system formed by discontinuous endothelial cells and remain in the blood. Inspired by the manipulation of biological behaviors via altering the mechanical properties of nanomaterials, some research has attempted to avoid the clearance of nanomaterials and enhance their tumor accumulation by adjusting their elasticity. To further understand the effects of mechanical property on passive and active tumor targeting, silica nanocapsules (SNCs) with variable Young's moduli were synthesized, and soft SNCs showed less uptake by macrophages and an improved capability of passing through the biological barriers in the tumor (**Figure 1.9**).[143] Hence, soft SNCs were more likely to pass through the intercellular spaces and penetrate deeper, while stiff SNCs would be trapped and accumulated in the marginal areas.

Due to the excellent capability to deform and adapt to the complex biological environments *in vivo*, soft nanomaterials typically show less macrophage uptake and clearance by the filtration system,

endowing them with prolonged blood circulation time and effective tumor accumulation. These research findings demonstrate the potential of the mechanical properties of nanomaterials for modulating their *in vivo* behaviors and performance, allowing for more effective anticancer therapeutics. Considering the nature of metal nanomaterials, the mechanical properties of these engineered nanomaterials are usually adjusted by manipulating the properties of surface modifiers at the design stage, such as altering the ingredient and concentration of surface modifier, changing the types or amount of crosslinking agents, controlling the crosslinking degree of surface modifier, and adjusting the thickness or water content of surface modifier layer.

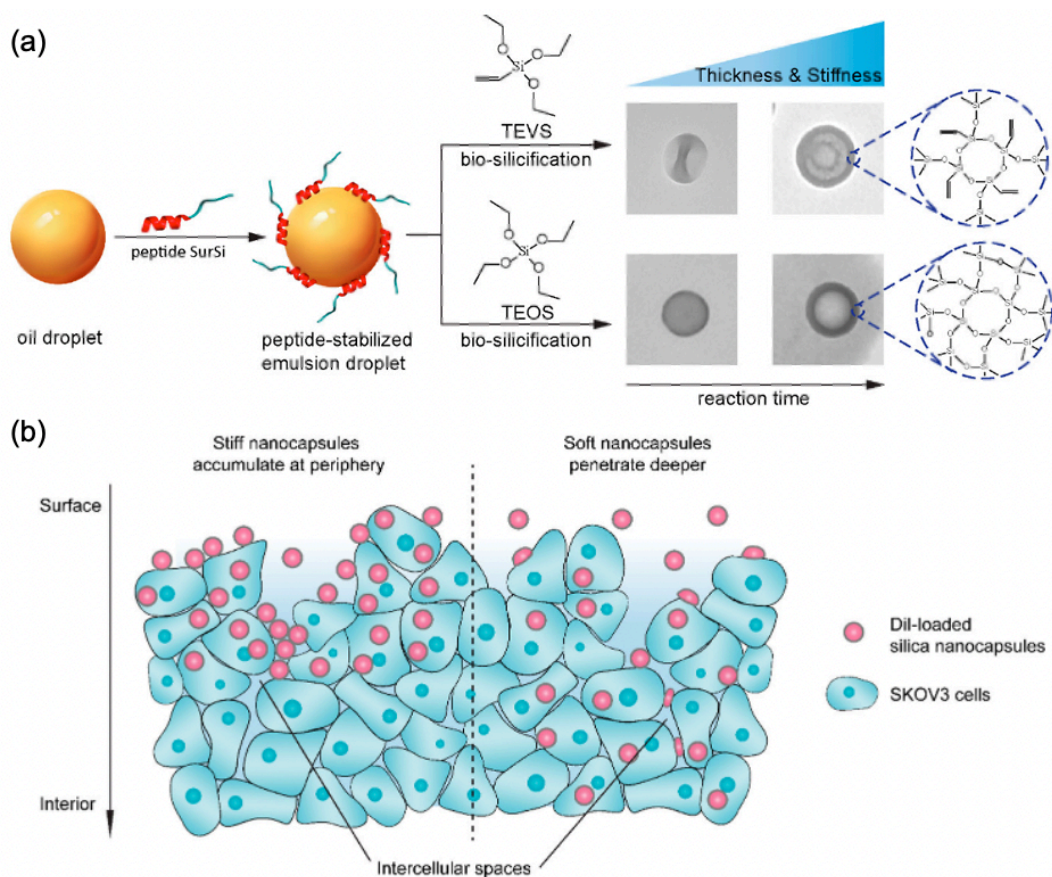


Figure 1.9. (a) Schematic illustration for the synthesis of silica nanocapsules having controllable mechanical properties. (b) Schematic illustration showing the penetration of stiff and soft nanocapsules in the tumor spheroids.

Adapted with permission from [143]. Copyright 2018 American Chemical Society.

1.2.4. Administration Routes

Since various administration routes lead to different fates, the administration routes of metal nanomaterials need to be considered at their design stage. Depending on the administration route, the *in vivo* behaviors and performance of metal nanomaterials can be significantly influenced, including their clearance, biokinetics, and biodistributions. Several administration routes are available in the clinic, each of which has its own benefits and drawbacks. It is critical to understand and assess how their implications affect the therapeutic outcome and patient experience.

1.2.4.1. Oral Route

The oral route is the most common and preferred option due to its ease of application and the convenience and compliance it provides for patients. An *in vivo* synthesis system by oral administration of raw materials was developed owing to the low risk of injection-caused infections, low systematic side effects, and straightforward usage.[50] Gold NPs can be synthesized *in vivo* by orally administering starting materials. At the initial stage, the level of gold NPs in the stomach approached a maximum value, indicating that gold NPs could be synthesized efficiently *in vivo*. The level of gold NPs in the stomach dropped significantly within the first 12 hours and steadily declined to near 0 within 72 hours, whereas the concentration in plasma began to rise and peaked at 10 hours and then fell within 24 hours. The concentration of gold in the liver and kidney rose rapidly and peaked at 24 and 18 hours before dropping to near 0 at 72 hours. The high concentration and rapid clearance rate of NPs in the two organs are advantageous for enhancing the therapeutic benefits while minimizing their toxicity. However, only a limited number of nanomaterial-based therapeutic agents have been reported thus far due to the challenges of oral administration, including the acidic condition in the stomach, the enzymes in the intestinal tract, as well as the interference from food. Owing to the capability to tackle the challenges in harsh conditions, metal nanomaterials are of great significance for enriching nanomedicine administration routes in clinical therapies.

1.2.4.2. Parenteral Route

The parenteral route includes intravenous, intramuscular, subcutaneous, and intradermal injections. Parenteral administration generally functions more rapidly than oral administration. Moreover, parenteral administration is nearly 100% bioavailable and can be therefore used to deliver therapeutic agents that may be poorly absorbed or ineffective when administered orally. Intravenous injection is the most commonly practiced route, but as described above, the nanomaterials delivered via direct intravenous injection may be cleared by the kidneys, liver, or MPS. It is possible to circumvent the limitations of intravenous injection with intramuscular, subcutaneous, and intradermal injections, but the injection location must be carefully considered for the optimal effect. In addition, nanomaterials could be injected directly into tumor tissue if the tumor is ideally located and easily accessible, which appears to be the most promising approach for delivering and retaining the desired dose of nanomaterials in patients.

1.2.4.3. Inhalation

Inhalation is another direct route for administering nanomaterials. For instance, nebulized ultrasmall multimodal gadolinium-based contrast agents (CAs) were developed as a noninvasive tool for the *in vivo* detection of non-small-cell lung cancer.[144] Inhalational administration of a low dose of the gadolinium-based CAs can significantly promote a high signal enhancement and improve the contrast-to-noise ratio, reducing toxicity to tissues and interobserver variability. For the first time, this work emphasizes the superiority of inhalation over conventional administration routes in investigating the pathomorphological changes caused by lung cancers. However, the nanomaterials deposited in the large airways would be cleared by the mucociliary escalator. Inhaled nanomaterials that reach the gas-exchange region, on the other hand, will be removed more slowly by alveolar macrophages or dendritic cells.[62] As a result, all these mechanisms of interaction and clearance by the respiratory system must be considered while designing new nanomaterials intended for inhalational administration.

Overall, in order to optimize and maximize the predesigned function of metal nanomaterials, the ideal design of metal nanomaterials in terms of size, shape, surface modification and functionalization, as well as administration route should allow the maximum accumulation of metal nanomaterials in the target sites (tumors), while ensuring minimal distribution in neighboring healthy tissue during RT.

1.3. Applications of Metal Nanomaterials in Radiation Therapy

1.3.1. Radiosensitization

For several decades, it has been recognized that high atomic number (high-Z) materials, especially metals, can facilitate radiation dose enhancement. A comprehensive understanding of the mechanisms underlying radiosensitization by metal nanomaterials at the tissue, cellular, and even molecular and genetic levels is essential for their design and clinical applications. The X-ray mass attenuation coefficient difference between high-Z nanomaterials and soft tissues allows high-Z nanomaterials to absorb more X-ray energy and achieve effective radiation dose deposition within tumors. High-Z nanomaterials absorb photons when irradiated with X-rays, leading to the photoelectric effect and Compton scattering through the interaction between photons and high-Z nanomaterials. As shown in **Figure 1.10**, when the photoelectric effect occurs, excess energy will generate and release in the forms of Auger electrons and fluorescent photons due to the ejection of photoelectrons and the filling of resulting vacancies by electrons from higher orbits. The electrons generated during these processes, i.e., photoelectrons, Compton electrons, and Auger electrons, can damage DNA directly or interact with water to produce ROS, which would then induce the apoptosis of cells in an indirect manner. Additionally, radiosensitization can be accomplished with high-Z nanomaterials by regulating the TMEs or manipulating cell behaviors (e.g., cell cycle and cell apoptosis).[145-147] For example, as described above, the concentration of GSH in tumor cells is higher than that in normal tissues. The GSH levels in tumor cells are as high as 0.5-11.6 mM, while GSH is present in normal tissues at extremely low levels, typically from 2 to 20 μ M.[148, 149] Apart from repairing DNA,[150] GSH can also protect cellular components from damage by depleting ROS

during RT.[151] Zhang et al. attempted to address this issue by decreasing the intracellular GSH level using a radiosensitizer based on histidine-capped gold nanoclusters (gold NCs@His).[152] RT can be thus enhanced as gold NCs@His could prevent the generated ROS from being consumed by GSH. Gold NCs@His was likewise found to cause the accumulation of cells in the G2/M phase, the most sensitive cell cycle stage to radiation, and reverse the radiation tolerance of tumor cells.[153]

In this section, recent advances in the metal nanomaterial-based radiosensitizers for RT enhancement, including gold (Au, $Z = 79$), platinum (Pt, $Z = 78$), silver (Ag, $Z = 47$), gadolinium (Gd, $Z = 64$), hafnium (Hf, $Z = 72$), tantalum (Ta, $Z = 73$), tungsten (W, $Z = 74$), and bismuth (Bi, $Z = 83$), are summarized and discussed.

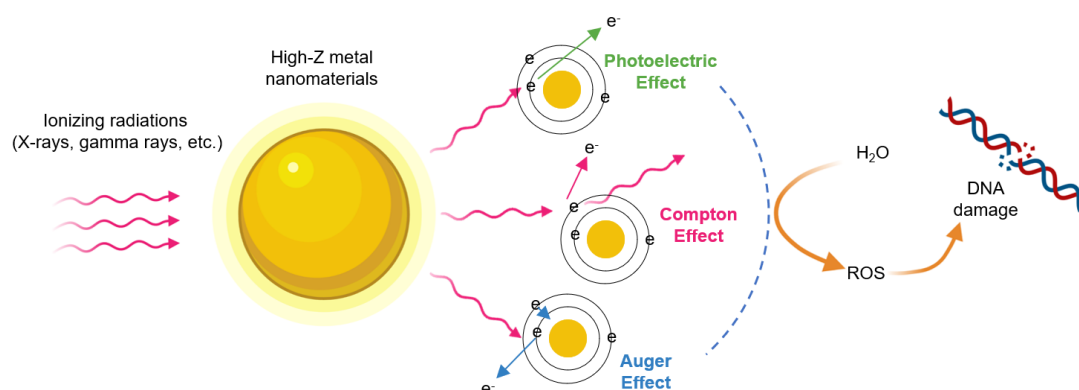


Figure 1.10. Illustration of radiosensitization via the interaction of X-rays and high-Z metal nanomaterials.

1.3.1.1. Gold

The excellent biocompatibility, low toxicity, and accessible surface functionalization of gold with different types and structures make them potential candidates in diverse biomedical fields, including clinical diagnostics, therapies, imaging agents, biosensors, and immunology.[127, 154-156] Particularly, gold nanomaterials have been identified as effective radiosensitizers due to their high X-ray absorption. A systematic evaluation of the biological efficacy of gold NPs distributed in the extracellular media for realistic cell geometries was conducted, in which radial dose distributions surrounding the gold NPs were determined at the nano scale.[157] The radiation dose-response of

cells surrounded by gold NPs was also investigated by varying (a) the cell shape, (b) the position of the nucleus within the cell, (c) the size of gold NPs, and (d) the photon energy. The sensitivity enhancement ratio (SER) increased by a factor of 1.20 when the nucleus was located close to the cell membrane in elliptical-shaped cells.

In addition to these parameters, radiosensitization is also dependent on the accumulation of gold nanomaterials, i.e., the amount of gold within the tumors. Lu et al. demonstrated the megavoltage (MV) radiosensitization of a human liver cancer line by combining gold NP-encapsulated microbubbles (gold MBs) with ultrasound.[158] Sonoporation is a technique that utilizes sound to temporarily alter the permeability of the plasma membrane of a cell, which has been used to improve the intercellular transfer of materials or genes. A sonoporation prior to radiation significantly boosted the intracellular concentration of gold NPs, which further enhanced the exposure to ionizing irradiation and promoted DNA damage, as evidenced by the higher levels of apoptosis markers and inferior cell surviving fractions after X-ray irradiation. Another work synthesized interbilayer-crosslinked multilamellar lipid vesicles (ICMV) containing amphiphilic gold NPs for an enhanced tumor accumulation, as amphiphilic gold NPs can enter live cells via both energy-dependent and energy-independent pathways without causing membrane poration or toxicity.[159] ICMVs could transport amphiphilic gold NPs to tumor cells, followed by endocytic ingestion of the lipid capsules. The subsequent breakdown of ICMVs within endolysosomes led to the release of gold NPs and their transfer to the internal membranes of target cells. The enhanced RT induced a threefold increase in cell damage due to the effective delivery and accumulation of gold NPs within cancer cells.

However, the nanomaterials that have entered tumors may still flow back into the bloodstream or diffuse into neighboring tissues, resulting in their depletion within tumors and an increase in adjacent tissues. A system based on the acid-triggered aggregation of gold NPs was therefore designed to serve as a radiosensitizer for enhancing RT (**Figure 1.11**).[156] The small-sized gold NPs (28.2 nm) were initially synthesized and then modified with two peptide-grafted 2,3-Dimethylmaleic anhydrides (DA),

denoted as gold NPs-A and NPs-B. Finally, the gold NPs system was achieved by evenly mixing gold NPs-A and NPs-B. Under normal conditions, gold NPs stayed dispersed and could penetrate into the tumor. After their penetration, the negative surface charge of gold NPs-B will be reversed into a positively charged state in response to the lower pH in the tumor by electrostatic interaction with negatively charged gold NPs-A, resulting in the formation of the gold NPs aggregates with larger sizes. After a 5-min incubation in a mildly acidic environment (pH 6.5), the size of the gold NPs system immediately increased from 32.6 to 912.8 nm, as shown in **Figure 1.11. (b)**. The resulting gold NPs aggregates exhibited long-term retention stability within tumors. More importantly, when exposed to gamma radiation, the gold NPs aggregates could generate more ROS to damage tumor cells, thereby enhancing the RT efficiency and obtaining a much higher SER value (1.73).

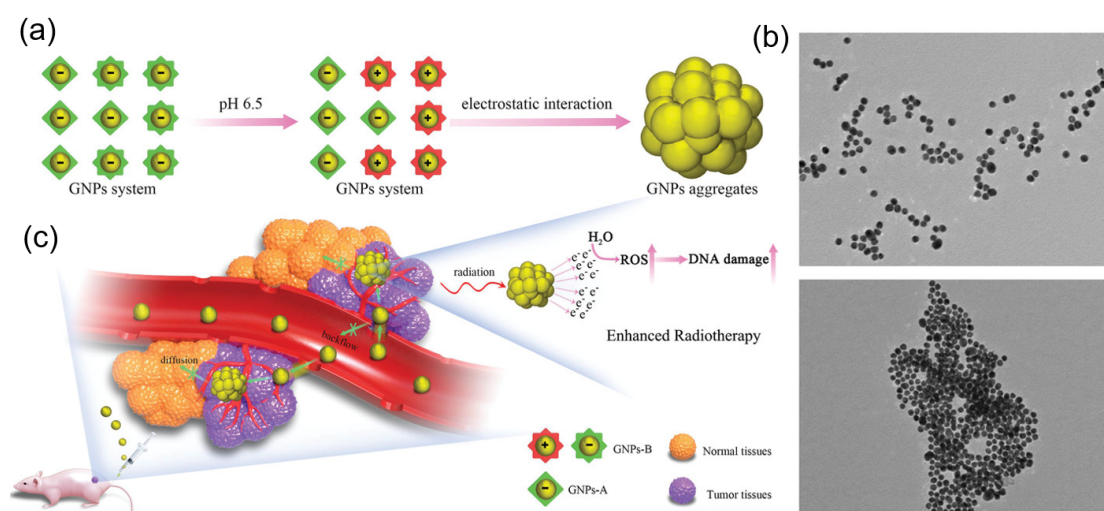


Figure 1.11. (a) Diagram depicting the acid-triggered aggregation and composition of the gold system. The gold NPs-B undergoes a charge reverse when exposed to an acidic environment, where electrostatic interaction further occurs with the gold NPs-A to form aggregates. (b) The TEM images of the gold NPs system at pH 7.4 and 6.5. (c) Schematic illustrations of *in vivo* behavior of gold NPs system after intravenous injection for increased tumor retention and enhanced RT.

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1.3.1.2 Platinum

Platinum-based therapeutic agents such as cisplatin, carboplatin, and oxaliplatin are well-known anticancer chemotherapy agents. Cisplatin has been proved effective as a radiosensitizer due to its capability to suppress DNA repair.[160, 161] Compared with gold nanomaterials, fewer works focus on using platinum nanomaterials as radiosensitizers, especially the synergistic effects of platinum nanomaterials on therapeutically relevant radiation. Daneshvar et al. synthesized platinum NPs for anticancer therapy in combination with 808-nm laser and X-ray irradiation.[162] Platinum NPs can cause the death of tumor cells by producing ROS in response to X-ray radiation. Due to the high penetration of laser in tissues, laser radiation prior to RT into cells containing platinum NPs allowed for deeper energy delivery and increased ROS generation. Hence the significantly enhanced RT had a superior therapeutic effect compared to sole X-ray radiation or laser illumination. To date, the majority of research has focused on ultrasmall platinum particles or clusters, while a few have examined larger and more complicated structures.[163, 164] In another work, PEGylated platinum nanoflowers with a more complex structure were synthesized as a radiosensitizer.[165] The unique structure can also allow easy functionalization with ligands such as drugs, radioisotopes, or enzymes to enhance RT efficiency, target delivery, or other diagnostic performance.

1.3.1.3 Silver

The antibacterial and antiviral properties of silver nanomaterials have been well established, and their applications as radiosensitizers for anticancer treatments are also explored more recently. In particular, apart from the radiation dose deposition and radiation-induced photoelectrons, Compton electrons, and Auger electrons, the underlying mechanism of radiosensitization with silver nanomaterials is also dependent on the autophagy enhanced by silver nanomaterials.[166] Liu et al. assessed the efficiency of silver NPs in the radiosensitization of hypoxic glioma cells (U251 and C6).[167] The SER values indicated that silver NPs exhibited a stronger radiosensitization effect in hypoxic cells (U251: 1.78; C6: 1.84) compared with normoxic cells (U251: 1.34; C6: 1.45). More

importantly, hypoxia U251 cells treated with silver NPs and X-rays produced more ROS than those treated with X-rays alone. The considerable destructive autophagy induced by the elevated ROS further caused increased apoptosis, demonstrating the potential of silver nanomaterials as a highly effective radiosensitizer. Gu et al. further evaluated and compared the radiosensitizing efficacies of silver NPs and gold NPs on glioma at clinical MV energies.[168] Silver NPs exhibited a greater radiosensitizing capacity than gold NPs at the same mass and molar concentrations, which promoted a higher apoptosis rate of tumor cells. Furthermore, when silver NPs were combined with radiation, autophagy levels were dramatically increased compared to the combination of gold NPs and radiation. The strongly upregulated autophagy level following the radiation with silver NPs indicated that the regulation of autophagy response could improve the eventual therapeutic outcome. Nevertheless, some studies claimed that silver nanomaterials might be toxic to stem cells,[169] liver,[170] and other organs,[171] which may explain the limited effort in developing silver-based radiosensitizers.

1.3.1.4 Gadolinium

Gadolinium chelates have been extensively utilized as T₁ MRI contrast agents due to the unique magnetic properties of the gadolinium(III) ions.[172] Gadolinium nanomaterials have also been identified as promising radiosensitizers for RT according to a number of studies, in which the radiosensitization efficacy and the safety of gadolinium nanomaterials were also evaluated.[173, 174] Nebulized gadolinium-based ultra-small rigid platforms (USRPs) were developed for multimodal imaging and radiosensitization.[175] Direct administration of USPRs via inhalation significantly boosted their pulmonary delivery and uptake by tumors, which enabled improved imaging identification of the tumor. Moreover, mice that received RT with USRPs had a longer survival time (112 days) compared to the mice that were only treated with RT alone (77 days), confirming the sufficient radiosensitizing effect induced by gadolinium-based nanomaterials. The radiosensitization of gadolinium NPs was further investigated in a study by Rima et al., and two major cellular internalization routes of gadolinium NPs, passive diffusion and micropinocytosis, were also proved

and quantified.[176] While passive diffusion of gadolinium NPs was insufficient to induce a significant radiosensitization *in vitro*, the gadolinium NPs internalized via micropinocytosis showed an enhanced radiosensitization efficacy over a narrow range of particle incubation concentrations. Although gadolinium has a lower atomic number compared to gold and platinum, it is sufficient to induce dose enhancement in RT. More importantly, gadolinium nanomaterials are appealing as they can be easily monitored using MRI. Monitoring biodistribution via MRI was critical for optimizing therapeutic effect since RT can be initiated only when the gadolinium level is high in the tumor and low in the surrounding tissue.[177]

1.3.1.5 Hafnium

Hafnium is another high-Z element that has been explored as radiosensitizers, and Hf-based nanomaterials for radiosensitization are typically in the form of oxides. For example, a nanoplateform based HfO₂ NPs was constructed for the radiosensitization and controlled release of anticancer drugs under specific conditions.[178] The nanoplateform was capable of accumulating inside the cells and potentiating the RT efficacy, as evidenced by the long-term cell viability, in which the vitality of irradiated colon cancer cells treated with bare HfO₂ NPs reduced more rapidly than other groups. It is of interest that NBTXR3, a novel radiosensitizer composed of functionalized HfO₂ crystalline NPs, has received European marketing approval for soft tissue sarcoma and its global phase III clinical trials for head and neck are about to be launched. NBTXR3 received FDA fast track designation in 2020 for the treatment of patients with locally advanced head and neck squamous cell carcinoma (LA-HNSCC) who are not eligible for platinum-based chemotherapy. It has been shown to improve survival in elderly and frail patients with LA-HNSCC. The best observed objective response rate (ORR) for target lesions was 83.9%, while the best observed complete response rate (CRR) for target lesions was 67.7%, based on a phase I trial with 43 participants.[179] In a frail population with a significant disease burden, the treatment was shown to be safe and well-tolerated. NBTXR3 is

currently being examined in different cancers, including pancreatic cancer, lung cancer, and esophageal cancer.

1.3.1.6 Tantalum

The superior biocompatibility and nontoxicity of tantalum have led to its widespread applications in medical implants, artificial joints, and stents. Tantalum oxide has also been attempted to be used as a radiosensitizer due to its strong X-ray attention ability. Peng et al. fabricated PEGylated oxygen-carrying hollow mesoporous TaOx nanospheres modified with CuS NPs (HMTCP) for triple sensitization of radiotherapy.[38] Because of the interaction of tantalum with X-rays, HMTCP had an inherent radiosensitization effect, which could enhance X-ray-induced DNA damage during RT. The release of oxygen from NIR laser-triggered moderate hyperthermia might boost radiotherapeutic efficiency even further. The as-prepared HMTCP can achieve nearly complete tumor growth suppression with no evident adverse effects, opening new avenues for multisensitizing tumor RT.

1.3.1.7 Tungsten

Transition-metal dichalcogenides, e.g., tungsten disulfide (WS₂), have recently attracted wide attention owing to their unique structures and physical/chemical properties. It has been reported that WS₂ nanomaterials are capable of serving as radiosensitizers.[180, 181] Interestingly, Chao et al. demonstrated the use of WS₂ nanoflakes for self-sensitized radioisotope treatment.[182] ¹⁸⁸Re is a common IRT radioisotope that emits both beta and gamma rays and has a strong binding with oxygen and sulfur atoms. ¹⁸⁸Re ions can be directly labeled on PEGylated WS₂ nanoflakes through the chelator-free approach to build ¹⁸⁸Re-WS₂-PEG. ¹⁸⁸Re-WS₂-PEG exhibited an excellent radiolabeling stability and the IRT could be enhanced via self-sensitization based on the interaction between WS₂ nanoflakes and the ¹⁸⁸Re anchored on their surface. ¹⁸⁸Re-WS₂-PEG accordingly showed a dramatically higher tumor cell killing efficacy than free ¹⁸⁸Re due to the effective uptake of ¹⁸⁸Re after binding to WS₂-PEG and the self-sensitization property.

1.3.1.8. Bismuth

Although still in their infancy, bismuth-based nanomaterials have recently attracted much attention due to their outstanding properties, such as high biostability, easy functionalization, high X-ray attenuation coefficient, strong NIR absorbance, and high photothermal conversion efficiency. Compared to other metals, bismuth is one of the most biocompatible and biofriendly high-Z metals, rendering it more appropriate for *in vivo* applications. Bismuth-based nanomaterials, e.g., simple substance (Bi), bismuth oxyhalides (BiOX, where X is Cl, Br, or I), bismuth oxide (Bi₂O₃), bismuth sulfide (Bi₂S₃), bismuth selenide (Bi₂Se₃), exhibit excellent capabilities in enhancing CT contrast and RT efficacy. Deng et al. described the use of folate functionalized RBC membranes for the modification of bismuth NPs (F-RBC bismuth NPs) to enhance the RT efficacy in breast cancer treatment.[183] Compared to other bismuth nanomaterials, F-RBC bismuth NPs demonstrated promising blood retention and tumor accumulation capacities due to the cell membrane coating and folate-enabled targeting capability, which can considerably inhibit tumor growth and improve the survival rate in mice. Under physiological conditions, the cell membrane modification and dissolution behavior of bismuth NPs allowed the fine tuning of the circulation, radiation enhancement, and clearance, thereby maximizing the treatment effect and minimizing long-term toxicity.

1.3.2. Imaging

In the clinic, imaging plays a vital role in preoperative diagnosis, intraoperative monitoring, as well as the post-treatment assessment of therapeutic outcomes. Imaging provides the surgeons with detailed information about the lesion as a basis for the diagnosis and treatment throughout the perioperative period, which helps relieve symptoms of patients and improve their survival rate. For example, during image-guided radiation therapy (IGRT), a high-precision anticancer treatment, high-dose radiation can be delivered to target lesions with the guidance of advanced and powerful imaging techniques, such as CT, MRI, optical imaging, and photoacoustic (PA) imaging. CAs are commonly

used to assist surgeons in acquiring high-quality images of the target region, especially for the soft tissues that are in contact with body fluid, by enhancing the contrast between the target and surrounding tissues. Due to the high atomic number and excellent X-ray attenuation coefficient, metal nanomaterials are clearly competitive candidates for current CAs, such as iodine-based CAs and barium sulfate. In this section, metal nanomaterials employed as CAs in various imaging technologies, including CT imaging, MRI, optical imaging, PA imaging, and multimodal imaging, will be presented and summarized (**Table 1**).

1.3.2.1. CT Imaging

CT imaging is a revolutionary tool for noninvasive diagnostic imaging since it provides 3D imaging with high spatial resolution and tissue penetration. Targets with varying X-ray attenuation or densities can cause different contrast during the imaging process. Customized imaging protocols are accordingly required for enhanced contrast and image quality when the target region has a similar density or X-ray attenuation. Metal nanomaterials have higher contrast enhancement than iodine-based CAs, the most commonly used CAs in clinical practice, at the same concentration. Therefore, there has been growing interest in developing metal nanomaterial-based CT CAs. Gold nanomaterials are effective CAs due to their prolonged blood circulation time and increased CT sensitivity. For example, Kim et al. found that PEG-functionalized gold NPs exhibited a 5.7 times higher X-ray attenuation intensity than Ultravist, an iodine-based CA.[184] Similarly, Lin et al. reported a controlled construction of gold NPs *in situ* from β -cyclodextrin-derived unimolecular micelles for CT imaging.[185] The reducing groups of PDMA block in β -cyclodextrin allowed the *in situ* reduction of gold ions and the decoration of formed gold NPs with micellar PDMA block, which enabled a narrow size distribution and effectively prevented gold NPs from aggregation. At 800 μ M, the X-ray attenuation intensity of β -cyclodextrin-gold NPs was approximately 37% higher than that of Omipaque, another conventional iodine-based CA. An advantage of iodine-based CAs is their short circulation half-lives since it minimizes potential damage and toxicity. As previously discussed, while

small gold NPs (diameter < 5 nm) can be rapidly cleared via the kidneys, they lack the efficient accumulation in the target lesion, which is vital for the subsequent acquisition of high-quality CT images. In order to facilitate both effective CT imaging and complete clearance via the kidneys following imaging, a platform (gold-PCPP) consisting of a biodegradable polyphosphazene-based polyacid (PCPP) nanospheres and excretable gold NPs (diameter < 5.5 nm) was proposed (**Figure 1.12**).^[186] With a tunable size range (40-500 nm), gold-PCPP can accumulate at the target region, fulfill its role as a CT CA, and then degrade into harmless byproducts, ultimately allowing the released gold NPs to be rapidly cleared via the kidneys after imaging.

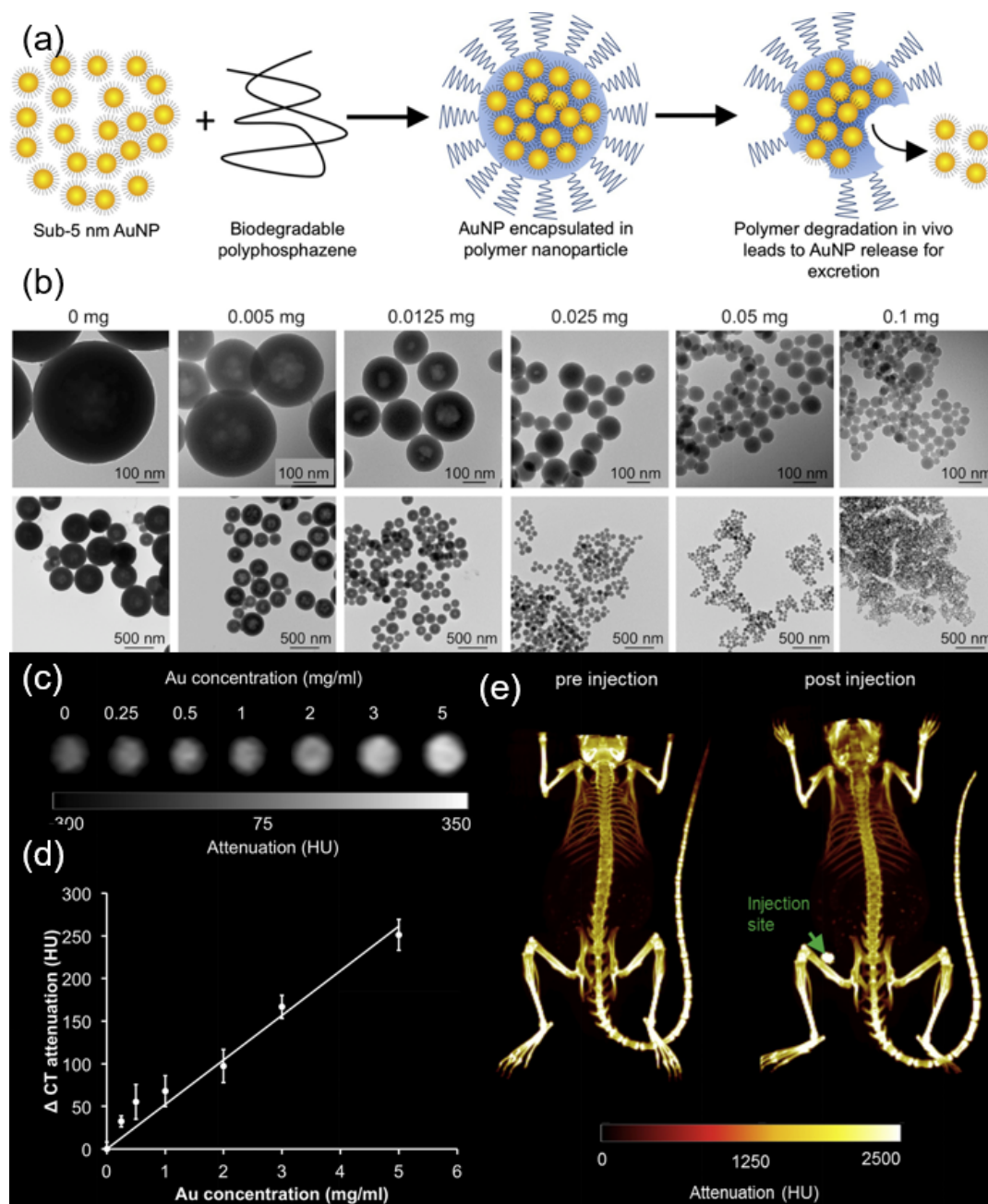


Figure 1.12. (a) Schematic depiction of biodegradable gold nanoparticles. (b) TEM images of PCPP nanoparticles synthesized using a microfluidic device with varying amounts of PEG-PLL added in the formulation. (c) CT image of gold-PCPP phantoms. (d) Linear correlation of CT attenuation and gold concentration in gold-PCPP in phantoms. (e) Strong CT contrast of gold-PCPP from intramuscular injection in mice.

Adapted from [186] with permission from Elsevier.

Platinum nanomaterials have unique advantages in absorbing light with wavelengths beyond 1000 nm compared to gold nanomaterials, which make platinum nanomaterials more superior candidates as CAs especially when CT imaging is required for the multimodal imaging with PA imaging or the conjunction with photothermal therapy. Ma et al. prepared uniform worm-like platinum particles (platinum NWs) for CT imaging and photothermal therapy.[187] Platinum NWs exhibited a superior contrast performance in an *in vivo* model compared with the commercial product (iopromide) in the cross-sectional view and 3D reconstructed images. Moreover, with high optical absorption in the NIR-II region of the platinum NWs, the mild heating provided by the low-power 1064-nm laser and the NWs significantly improved the oxygen supply in the tumor region. Together with RT, remarkable tumor inhibition and minimal damage to surrounding healthy tissues were realized in the anticancer treatment.

The use of other high-Z elements as CT CAs is also widely investigated. For example, according to FitzGerald et al., the image contrast of gadolinium increased by 56%, 66%, and 72% compared to clinically used iodine at tube potentials of 100, 120, and 140 kVp, respectively.[188] Ytterbium NPs also demonstrated much higher efficacy than a clinical iodine agent, and they could be even more efficient than gold-, platinum-, bismuth-, and tantalum-based CT CAs under normal operating conditions (120 kVp).[189] An important contributing factor to the improvement is the K-edge energy of ytterbium (61 keV), which located within the higher energy region of the X-ray spectrum. In clinical CT imaging, the use of a range of high-Z elements has advantages over iodine in designing CAs for enhancing image contrast, which can further improve image quality, provide detailed information about the lesion, and ultimately reduce radiation dose and contrast agent dosage for minimal damage. On the other hand, the almost negligible biotoxicity of iodine-based CAs is the primary reason for their widespread usage in the clinic. Therefore, in addition to focusing on the superior performance the metal nanomaterial-based CAs exhibit over iodine-based agents, the

biocompatibility and clearance pathways of the metal nanomaterial-based CAs also require attention during their design to ensure their biosafety.

1.3.2.2. MRI

A number of advantages have contributed to the extensive use of MRI, including its noninvasive nature, high spatial resolution, high contrast of soft tissues, deep penetration, and abundance of pulse sequences. An MRI image can usually be improved in two ways: using advanced pulse sequences and hardware or introducing CAs.[190] The seven unpaired electrons on the outer layer of gadolinium enable its paramagnetic property, making Gd(III) chelates the most widely used CAs for MRI in the clinic, including gadoterate meglumine, gadoxetate disodium, gadobutrol, gadopentetate dimeglumine, gadobenate dimeglumine, gadodiamide, gadoversetamide, gadoteridol.[191] With the presence of gadolinium-based CAs, water molecules can shorten their longitudinal proton relaxation time (T_1), which increases the signal intensity and causes the bright signals in the images. However, as a result of the nonspecific distribution and moderate longitudinal relaxation rate of these gadolinium-based CAs, a high-dose intake is usually required, which might lead to potential side effects, including longer circulation time in the blood, increased background, and higher accumulation/deposition of excess ions in healthy tissues. Patil et al., therefore, designed gadolinium conjugates with antibodies and peptides as nano CAs. Compared to the commercial CA MultiHance, the novel targeted nano CAs with star-PEG features displayed greater relaxivity and contrast, providing a suitable time window for clinic tumor diagnosis. However, Gd(III) leakage is a matter of concern, particularly since free Gd(III) may cause nephrogenic systemic fibrosis.[192] Alternatively, iron-based CAs are preferred since iron has better compatibility with organisms. As demonstrated in earlier studies, magnetic iron oxide (Fe_3O_4) NPs have a remarkable T_1 enhanced contrast effect in the small form and a substantial T_2 enhanced contrast effect in an assembly or larger-structure form.[193-195] Lin et al. utilized pH- and GSH-responsive zeolitic imidazole frameworks (ZIFs), a biocompatible delivery carrier constructed by zinc ions and imidazole ligands, as a matrix to assemble small Fe_3O_4 NPs (Fe_3O_4 -ZIF).[196] Under

physiological circumstances, Fe_3O_4 -ZIF first demonstrated the T_2 contrast-enhancing capability. Subsequently, the acidic environment and the overexpression of GSH within the tumor would trigger the disassembly of Fe_3O_4 -ZIF and the release of small Fe_3O_4 NPs, converting the darkening T_2 contrast to brightening T_1 signals. Such T_2 - T_1 switching CA is more preferable in clinical diagnostics, as it could allow for brighter imaging with higher sensitivity and signal-to-noise ratio and provide more comprehensive information on target lesions.

1.3.2.3. Optical Imaging

In optical imaging, it is typical to monitor biological information about cells and tissues using fluorescent probes. Fluorescence imaging is known for its high imaging contrast, high sensitivity, and targeting capabilities, which makes it a powerful tool for monitoring tumors or the distribution and metabolism of administered drugs. NIR fluorescent dyes, e.g., cyanine, phthalocyanines and porphyrin derivatives, squaraine derivatives, xanthenes, and benzo[c]heterocycles, are appropriate for fluorescence imaging.[197] Inorganic nanomaterials display much higher colloid and photostability than organic dyes under physiological conditions. For instance, upconversion NPs (UCNPs) hold great potential in biomedical imaging since they can emit visible light under NIR irradiation.[198, 199] Moreover, metal nanomaterials have also displayed excellent fluorescence as optical imaging agent candidates. Gold nanoclusters (gold NCs), as an example, have been receiving much attention recently for their strong luminescence. Li et al. used the pea protein isolate (PPI), an abundant and sustainable plant protein, as a reducing and stabilizing agent to facilitate produce gold NCs (gold NCs/PPI).[200] Gold NCs/PPI were successfully used as a bioimaging probe in the tumor after being coated with RBC membranes. Huang et al. demonstrated the use of 4-PyET or 2-PyET in synthesizing gold clusters. The newly developed family of basic gold clusters and their basic Py-terminated feature are promising for expanding their applications in biomedical imaging.[201, 202]

1.3.2.4. PA Imaging

PA imaging is a unique imaging technique based on the photoacoustic effect. It is derived from ultrasound signals produced by the thermoelastic expansion caused by the absorption of the pulsed laser by the target biological tissue. Due to the minimal acoustic scattering in PA imaging, the high optical contrast and resolution allow PA imaging to overcome the fundamental depth limitation.[203] PA imaging is capable of mapping the oxygenation state of hemoglobin or the distribution of different chemicals *in vivo* by adjusting the incident light to the absorption band of specific molecules.[204] The tunable optical properties and acceptable signal-to-noise ratio of gold nanomaterials have led to their widespread use as PA imaging agents. Ye et al. presented the synthesis of gold nanotubes (gold NTs) with controlled lengths ranging from 300 to 700 nm.[205] The well-defined shape endowed the resultant gold NTs with strong SPR-based absorption in the NIR region and excellent *in vivo* photoacoustic conversion capability at lesions, demonstrating that gold NTs can be applied as a safe and effective theranostic system for PA imaging and photothermal therapy.

1.3.2.5. Multimodal Imaging

Accurate disease diagnosis has sparked significant concern because of its critical significance in precise treatment. On the other hand, single-modality imaging may not be sufficient for a comprehensive and high-precision tumor diagnosis. Consequently, combining complementary imaging modalities is indispensable for providing more comprehensive and accurate information as guidance for surgeons and patients. MRI/CT is a representative example of multimodal imaging. Luo et al. designed a novel nanocomposite, namely the $\text{Fe}_3\text{O}_4@\text{PDA}@BSA\text{-Bi}_2\text{S}_3$ composite, for the *in vivo* MRI/CT/thermal imaging (**Figure 1.13**).[206] The superparamagnetism of Fe_3O_4 NPs shortened the T_2 relaxation time of water protons, which led to a boost in MRI contrast and sensitivity. At the same time, Bi_2S_3 NPs strengthened the CT contrast owing to the high X-ray attenuation of bismuth atoms, synergistically affording the desirable precise diagnosis of cancer. Other multimodal imaging, such as PA/optical/MRI,[207, 208] optical/MRI/CT,[209-211] and optical/PA/CT, [212] have also

been adopted for a more comprehensive diagnosis. Typically, functional nanomaterials for multimodal imaging are constructed by encapsulating functional molecules or modifying them after synthesis, which would be time-consuming, complex, and difficult to reproduce. A bottom-up synthesis of metal-ion-doped WS₂ nanoflakes for multimodal imaging was proposed by Liang et al. to simplify these procedures.[213] The WS₂:Gd(III) was synthesized using a high-temperature solution method by reacting WCl₆ and GdCl₃ with sulfur. WS₂:Gd(III) exhibited excellent multimodal imaging performance since WS₂ had a strong NIR absorbance and X-ray attenuation ability for PA imaging and CT, while Gd(III) doping offered a paramagnetic property for MRI.

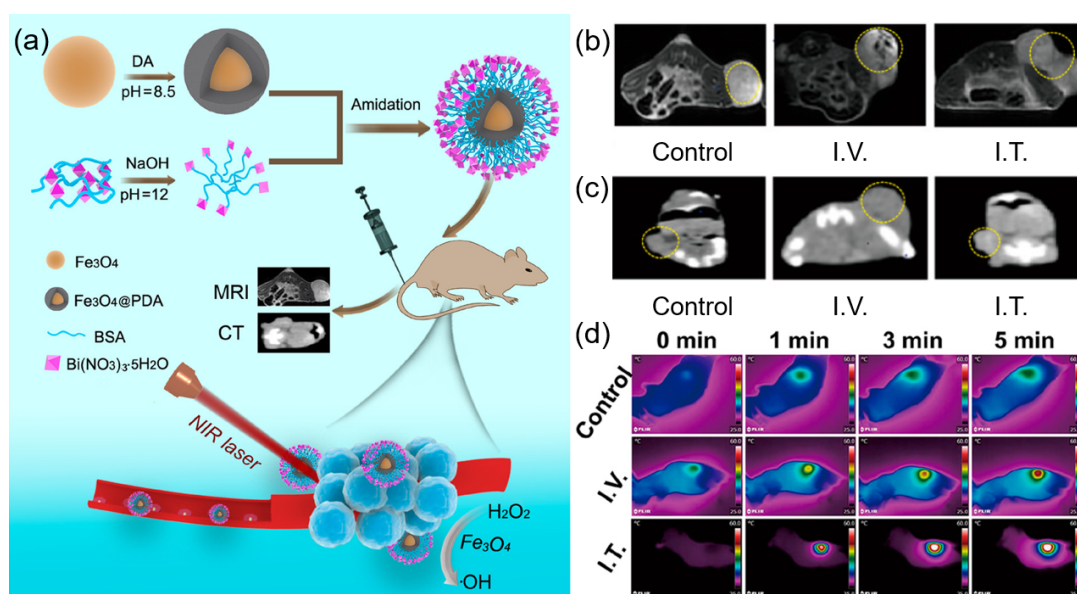


Figure 1.13. (a) Schematic Illustration of the Stepwise Synthesis of Fe₃O₄@PDA@BSA-Bi₂S₃ composite for the *in vivo* MRI/CT imaging. (b) Tumor MRI pictures of the different treated mice. (c) *In vivo* CT images of the different treated mice. (d) Thermal imaging of mice after laser irradiation at preset time points.

Table 1.1. Summary of metal nanomaterials for imaging applications.

Imaging modality	Energy source	Imaging depth	Advantages	Disadvantages	Metal nanomaterials	Other functions
CT imaging	X-ray	no limit	high spatial resolution	low sensitivity	gold NPs[184, 185]	chemotherapy
			high tissue penetration	low soft tissue contrast	gold nanostars[155]	photodynamic therapy
			short imaging time	ionizing radiation	platinum nanoworms[187]	photothermal therapy
					gadolinium[188]	image-guided therapy
MRI	radiofrequency magnetic field	no limit	high spatial resolution	low sensitivity	ytterbium NPs[189]	
			high tissue penetration	long imaging time	bismuth nanotubes[214]	
			high soft tissue contrast	long processing time	bismuth sulfide [215, 216]	
				high cost	gadolinium NPs[217, 218]	ROS amplification
Optical imaging	visible light or NIR	NIR-I: 5-8 mm NIR-II: < 4 cm	high sensitivity	long imaging time	gadolinium oxide[219, 220]	chemodynamic therapy
			real-time imaging	long processing time	iron oxide[194, 195, 221-223]	photothermal therapy
			low cost	high cost	iron sponge[224]	magnetolytic therapy
				probes may be needed		
PA imaging	pulsed laser	< 8 cm	high sensitivity	low spatial resolution	gold nanoclusters[200, 225-227]	tumor ablation
			high spatial resolution	poor tissue penetration		photodynamic therapy
			high tissue penetration	not available for some organs		
					UCNPs[204]	photodynamic therapy
Multimodal imaging	multi sources	no limit	complementary imaging	PA contrast agents needed	gold nanotubes[205]	photothermal therapy
			comprehensive diagnosis	CAs needed	mesoporous platinum[228]	
					gold NPs [186, 209, 210, 229, 230]	MRI/CT
					bismuth[206, 231, 232]	PA/CT/thermal imaging
					iron[207, 233, 234]	MRI/CT/thermal imaging
						PA/MRI/optical imaging

1.3.3. Target Delivery

Extra therapeutic agents, such as anticancer drugs and internal radioisotope, can effectively enhance cancer treatment outcomes. However, these agents are also readily metabolized by the liver or cleared by the kidneys. Therefore, target delivery and less rapid clearance from the body are essential for these agents to function as intended. The use of metal nanomaterials, which are typically designed to endow them with tumor-targeting capability, for delivering therapeutic agents can be a promising strategy to increase the site-specificity and therapeutic index, thereby preventing potential multi-drug resistance and off-target biotoxicity.

1.3.3.1. Anticancer Drugs

The radiation dose is usually strictly limited to prevent RT damage to healthy tissues surrounding the tumor and to avoid potential risks, such as cardiac toxicity, secondary malignancies, radiation pneumonitis, and lymphedema. However, dose restriction will limit the therapeutic effect of RT, thus, combining RT and anticancer drugs is preferred for enhanced cancer treatment. In a selenium-doping tellurium nanorod-templated method, Cai et al. fabricated gold hollow nanorods (gold HNRs) with a controllable aspect ratio.[235] Such a structure allowed for a high loading capacity of DOX. Apart from loading on the surface of the gold HNRs, drugs could also be loaded in the cavities because of the hollow porous structure. The heat generated under NIR irradiation can immediately kill the cancer cells and promote the release of the drug, thereby resulting in a better efficacy than monotherapy. Other anticancer drugs (e.g., methotrexate (MTX), hydroxyurea (hydroxycarbamide), 5-fluorouracil (5-FU), docetaxel (Dtxl), tirapazamine (TPZ), and 6-mercaptopurine (6-MP)) can also be delivered by other metal nanomaterials with different structures.[236-240] Recently, there have also been studies attempting to control the release of drugs from metal nanomaterial delivery systems through external stimuli or energy in order to achieve controllable and on-demand drug release. A porous-gold ultrasonic nanowire motor with high surface area and variable porosity was designed by Gradilla et al. for high-efficiency drug loading and release. [241] The porosity of the nanomotors constructed from

well-controlled templates can be fine-tuned for effective drug encapsulation. The nanomotors can be driven by ultrasound, which offered an attractive and potential approach for rapidly delivering larger therapeutic payloads to predetermined destinations in a target-specific manner. Moreover, the nanoporous gold nanowires displayed a high absorption in the NIR region, and the NIR irradiation-induced heat could be exploited for a light-controlled release of the loaded drugs.

1.3.3.2. Radioisotopes

IRT is extremely effective in the treatments of metastatic tumors or certain types of cancer by placing radioisotopes in the body and giving radiation directly to cancer cells. The major problem of using radioisotopes for IRT is their leakage into normal tissues, which might reduce their efficacy and increase the risks of side effects. Due to the selective delivery property, metal nanomaterial-based delivery systems are explored to deliver radioisotopes to the target sites for minimal toxicity to normal tissues. Moreover, metal nanomaterial delivery systems containing radioisotopes can also be integrated with other therapies, such as photothermal therapy, chemotherapy, and immunotherapy, to enhance the final therapeutic effect by utilizing their inherent properties. Chen et al. presented a pH-sensitive multifunctional platform containing radiolabeled palladium nanosheets using a simple mixture of nanosheets and radioisotopes (^{131}I -palladium-PEG).[242] The labeling efficiency was higher than 98%, and, interestingly, the absorption of radioiodine was more stable in an acidic environment. TMEs are more acidic than normal tissues, ^{131}I -palladium-PEG was therefore more stable in the tumor, which contributed to the accumulation of ^{131}I -palladium-PEG within the tumor, as well as the desorption and following clearance of ^{131}I in other tissues. The potential of ^{131}I -palladium-PEG serving as a therapeutic platform for the combination of photothermal therapy and IRT was also validated by the remarkable synergistic effect in the anticancer treatment.

Though high-Z metal nanomaterials have been extensively demonstrated for the sensitization of EBRT, they have received less attention for their use as radiosensitizers in IRT. In addition to serving as delivery vehicles for radioisotopes, high-Z metal nanomaterials can also function as

radiosensitizers by absorbing radiation from radioisotopes and generating energy to enhance the IRT efficacy. Pei et al. designed radioisotopes (technetium-99m and lutecium-177) labeled gold nanoclusters ($^{99\text{m}}\text{Tc@gold NCs}$ and $^{177}\text{Lu@gold NCs}$) via the chelation between GSH and radioisotopes (**Figure 1.14**).[243] These radioisotopes labeled gold NCs could induce antitumor immunity by activating dendritic cells, while at the same time endowing the nanotherapeutic platform with self-sensitization property by absorbing gamma rays and emitting charged particles. More charged secondary particles were detected by liquid scintillation spectroscopy from $^{99\text{m}}\text{Tc@gold NCs}$, indicating the radiosensitization ability of $^{99\text{m}}\text{Tc@gold NCs}$. Compared with free radioisotopes, the combination of gold-enhanced self-radiosensitization and antitumor immunity rendered radioisotopes labeled gold NCs more effective in the treatment of spontaneously metastatic tumors, resulting in a remarkable improvement in the survival rate and lifetime of transgenic mice.

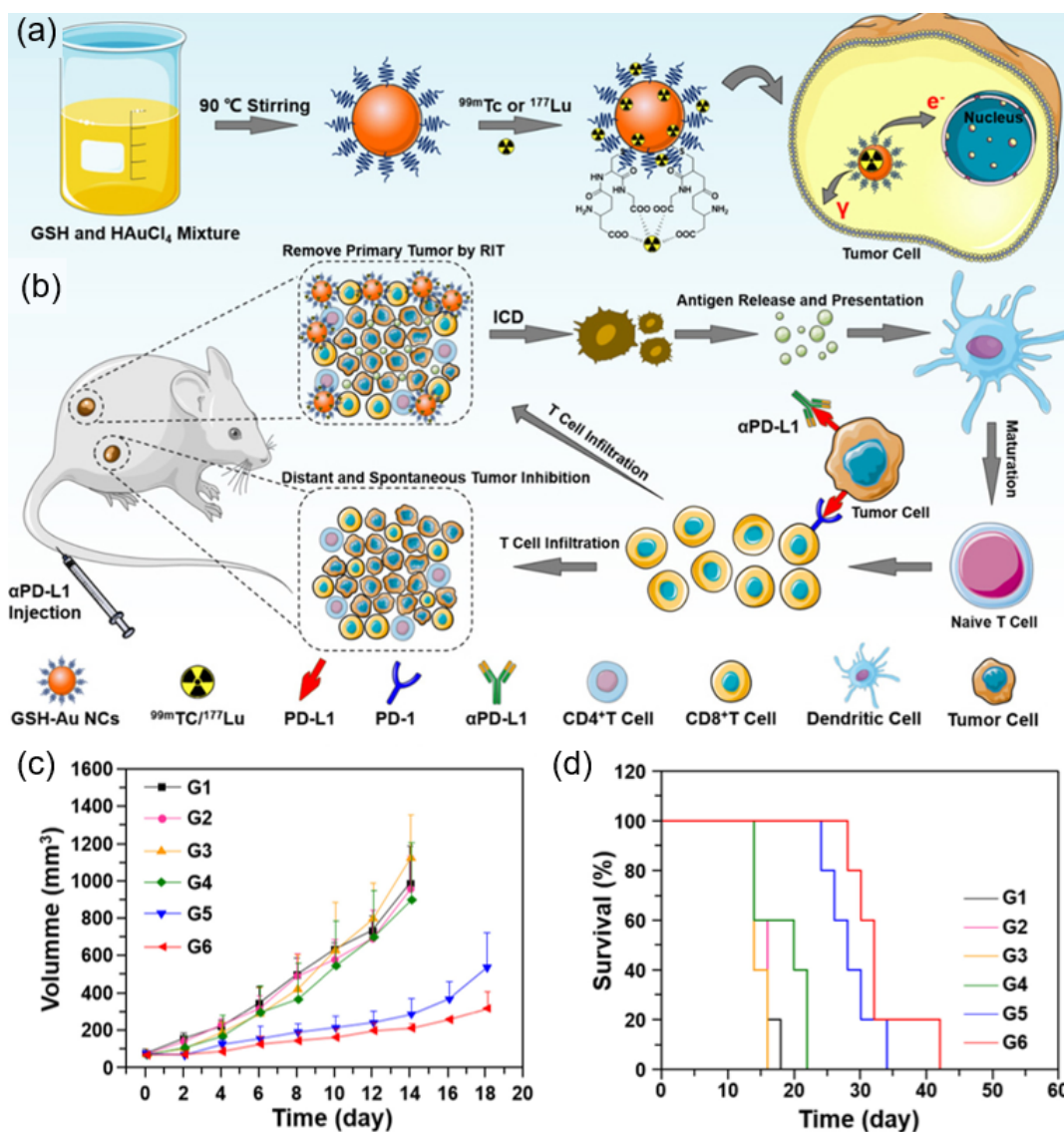


Figure 1.14. (a) Synthetic procedure of radionuclide labelled gold NCs. (b) The mechanism of antitumor immune responses induced by radionuclide labelled gold NCs in combination with checkpoint-blockade to inhibit distant and spontaneous tumors. (c) and (d) Distant tumor growth curves and survivorship curves of 4T1 tumor-bearing mice after various treatments.

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1.3.3.3. Oxygen

Instead of directly delivering therapeutic agents, oxygen delivery is a promising indirect approach for enhancing RT by modulating TMEs. The fact that tissues with a low oxygen supply will

be more resistant to radiation has been recognized for decades.[244] In a preclinical study, Gray et al. demonstrated for the first time that increased radiosensitivity was achieved by reducing hypoxia via oxygen delivery.[245] Perfluorocarbons (PFCs) are currently the most common nanomaterials utilized for loading and carrying oxygen. PFCs are a class of fluorocarbon-based chemical compounds in which fluorine atoms replace all or most of the hydrogen atoms. Oxygen-loaded PFCs are delivered to tumor tissues and release oxygen in response to oxygen pressure or other stimuli, thereby alleviating hypoxia in the tumor. As an example of delivering PFCs with metal nanomaterials, a nanomotor-based delivery system (motor-PFCs) that combined gold nanomotors with red blood cell membrane-cloaked PFCs was constructed for active intracellular delivery of oxygen.[246] Motor-PFCs presented a high dissolved oxygen level (around 1.5 mg/L) and can be driven by an acoustic field, which accelerated the intracellular delivery of oxygen to target cells. Under hypoxic circumstances, the oxygen loaded in the Motor-PFC was released sustainably. Ultrasound-driven motor-PFCs were also capable of releasing oxygen in a controllable way by varying the ultrasound intensity. Apart from ultrasound, other external stimuli, such as NIR laser, have been used to facilitate oxygen release from oxygen-loaded vehicles consisting of metal nanomaterials and PFCs (**Figure 1.15**).[38, 247] However, considering the tissue penetration capabilities of these external stimuli, exploring metal nanomaterial-based delivery systems that can respond to exogenous stimuli with larger penetration depths (e.g., magnetic fields or X-rays) might be more attractive and promising for the hypoxic RT of deep tumors.

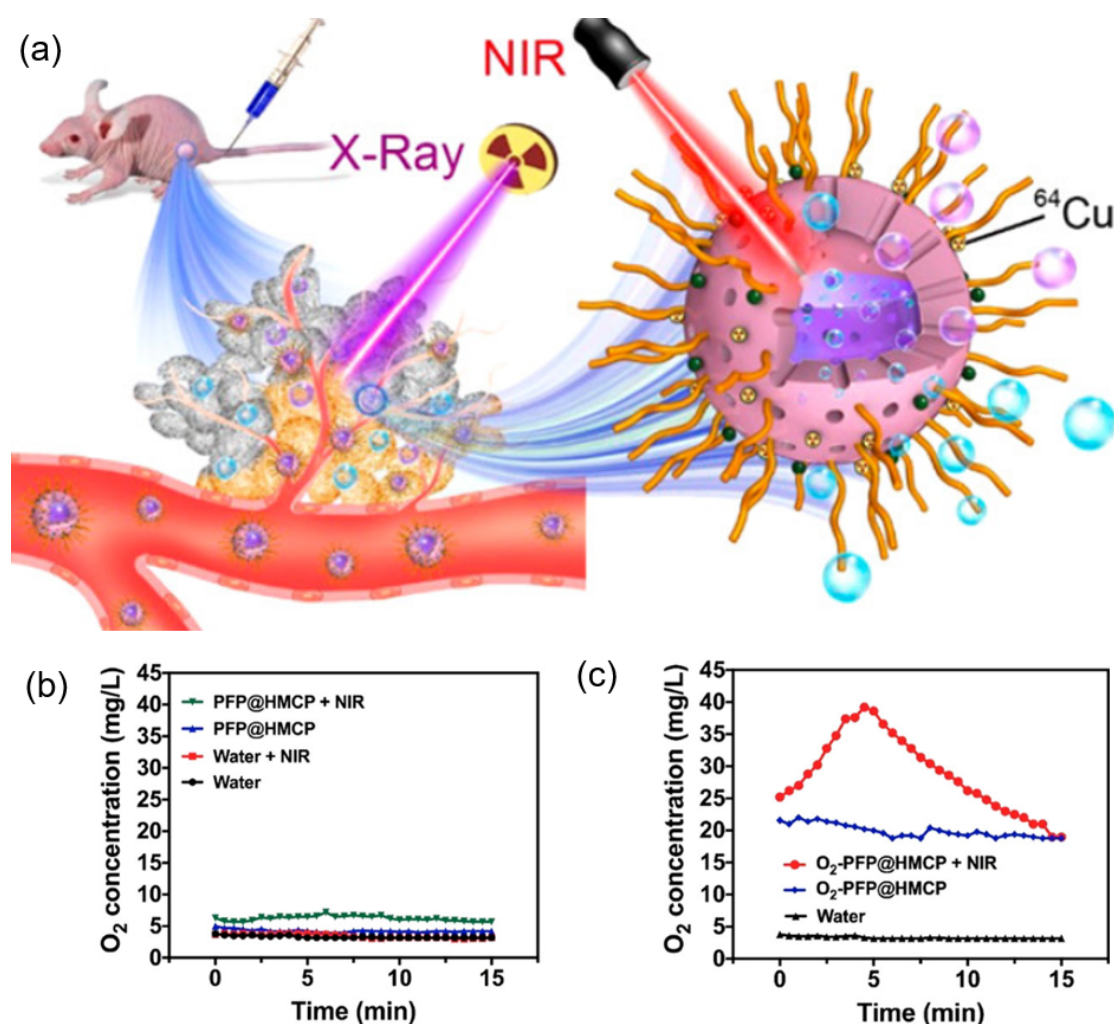


Figure 1.15. (a) Schematic of the mild-hyperthermia-induced PFP bubble release, and oxygen-sensitized RT. (b) Oxygen concentration changes of water after adding PFP@HMCP with or without NIR laser irradiation. (c) Oxygen concentration changes of water after adding oxygen-PFP@HMCP with or without laser irradiation.

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1.3.4. Relieving Hypoxia via Oxygen Generation

Due to the rapid and disorganized growth of tumors, the imbalance of oxygen consumption and supply within the tumor leads to tumor hypoxia. Hypoxia in tumor tissues will increase the level of hypoxia-inducible factor 1 (HIF-1) and upregulate vascular endothelial growth factor (VEGF) expression, which would further promote tumor angiogenesis.[248] The rapidly growing blood

vessels in hypoxic TMEs are usually abnormal and have tortuous shapes, irregular structures, fragmented or absent basement membranes, and increased permeability.[249] The resulting disruption in blood flow and dysfunction leads to an insufficient oxygen supply, further exacerbating tumor hypoxia.[250] Radioresistance in hypoxic tumor cells is three times higher than in aerobic cells.[251] As a result, radioresistance-induced tumor metastasis or recurrence will eventually cause the failure of RT. Although the production of some tumor metabolites in hypoxic TMEs, such as hydrogen peroxide (H_2O_2), might promote the mutagenesis and metastasis of cancer cells, they also provide opportunities for improving tumor hypoxia via oxygen generation *in situ*. By converting endogenous H_2O_2 to oxygen with porous platinum NPs, a new nanomedicine platform was proposed for simultaneously overcoming hypoxia-associated radioresistance and insufficient radiation energy deposition.[252] The high porosity and large surface area of platinum NPs ensured the sufficient penetration and contact of H_2O_2 molecules, facilitating the rapid conversion of H_2O_2 into oxygen. Porous platinum NPs could reverse TMEs from a hypoxic environment into a susceptible condition for efficient RT through its oxygen generating capability *in situ*. Similarly, metal nanomaterials with catalysis-like activity, e.g., gold clusters,[253] palladium@platinum nanosystem,[254] and gold@platinum nanopores,[255] can also behave as catalase to produce oxygen against hypoxia and the accompanying radioresistance.

There have also been studies seeking to enhance IRT by increasing oxygen levels and relieving hypoxic TME based on metal nanomaterial-based oxygen generators, in addition to their usage in EBRT. Pei et al. constructed a nano-oxygen generator consisting of metal-organic framework and gold-platinum, which could not only perform stable internal radiation but also efficiently generate oxygen to minimize the effect of hypoxia on IRT efficacy (**Figure 1.16**).[256] More importantly, a higher oxygen level boosted the antitumor immune response following IRT and promoted the infiltration of cytotoxic T cells into tumors. The activity of tumor cells was also declined by the relieved hypoxic microenvironment, which further slowed down the proliferation of tumor cells.

Owing to the above-mentioned dual effects, tumor metastasis can be effectively avoided after eliminating the primary tumor.

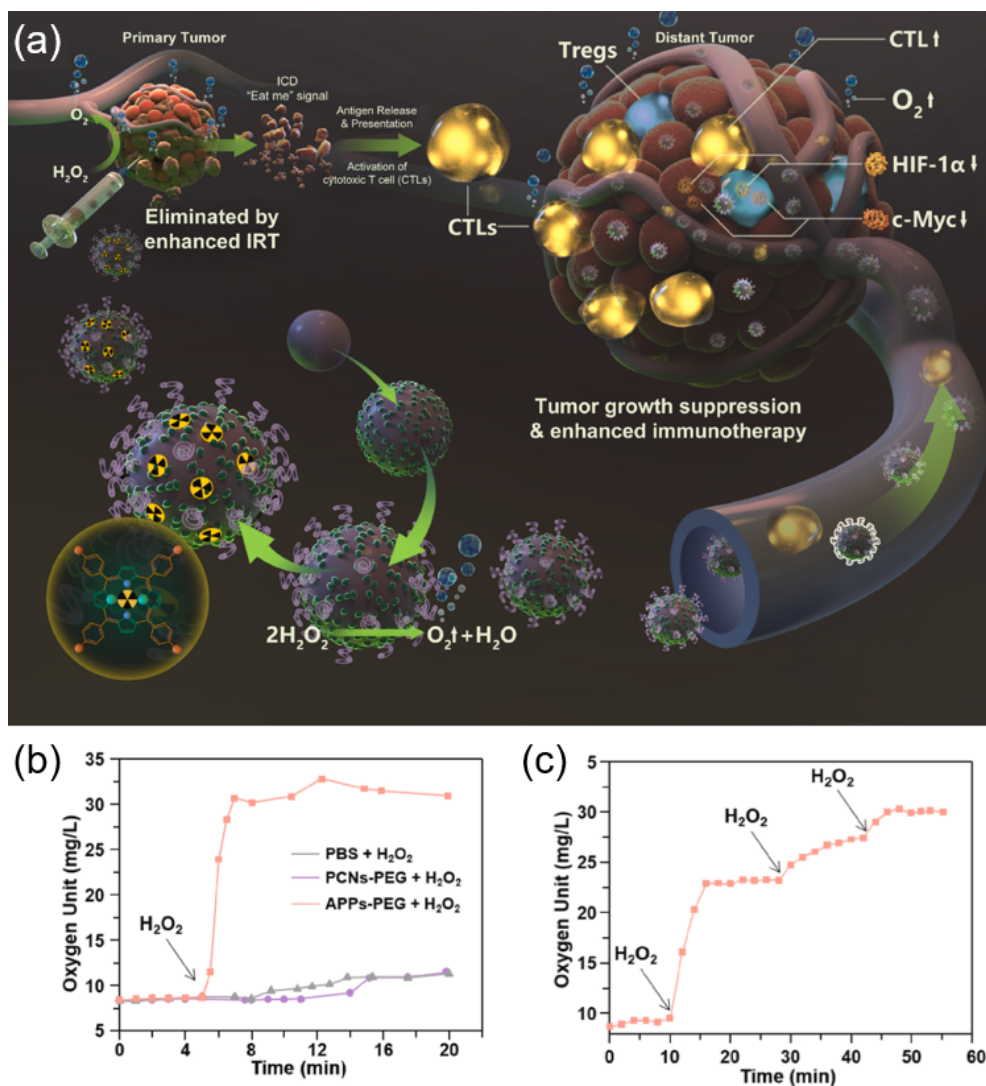


Figure 1.16. (a) Schematic diagram of radioactive nano-oxygen generator to enhance antitumor immunotherapy by regulating tumor microenvironment and metabolism. (b) Curve of oxygen generation after treated with PBS, PCNs-PEG and APPs-PEG at predesigned time points. (c) Repetitive oxygen generation after treated with APPs-PEG by repeated addition of H_2O_2 .

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1.3.5. Radioprotection

Severe injury and damage to surrounding healthy tissues are basically inevitable for patients due to the high energy ionizing radiation during RT, and the radiation dose is therefore limited to a certain level to avoid side effects. If the surrounding tissues are protected from the damage caused by X-rays, the radiation dose can be further increased to enhance the RT efficacy, while the side effects of RT can be minimized or avoided. Therefore, it would be an attractive strategy to develop radioprotectors to protect healthy tissues during RT. The indirect damage via the reaction between ROS and cellular macromolecules or other cellular components will destroy cell structure and induce cellular dysfunction, which ultimately caused the death of tumor cells. A common strategy for radioprotection is to decrease the indirect damage to normal cells by lowering the ROS level using radioprotectors during RT.

Depending on the mechanism, radioprotectors can lower ROS levels in three ways: (a) trapping of ROS, (b) redox reactions, and (c) catalysis-like (or enzyme-like) activity. [257] For example, fullerene (C₆₀) can trap radicals due to the electron affinity of its conjugated double bonds with unpaired electrons from ROS.[258, 259] Antioxidants, such as curcumin,[260, 261] bilirubin,[262] and polydopamine,[263, 264] have been shown to protect cells by scavenging ROS via redox reactions. The catalysis-like activities of some metal nanomaterials can also scavenge free radicals. In synthetic chemistry, platinum acts as a catalyst for hydrogenation and oxidation reactions. Therefore, platinum has the potential to lower ROS levels. The reducing activity of colloidal platinum NPs for H₂O₂ has been validated, [265] and their applications in scavenging ROS are further investigated.[266, 267] Jawaaid et al. found that pretreatment with platinum NPs can significantly decrease the production of radiation-induced ROS in human lymphoma U937 cells, indicating that platinum NPs can protect cells from apoptosis by inhibiting intracellular ROS.[268] In a recent study, platinum clusters have also been discovered to prevent DNA damage in irradiated cells.[269] The survival rate of irradiated mice increased to 30% after the treatment with platinum clusters. In contrast, all

irradiated mice without the protection of platinum clusters died after 15 days. By scavenging free radicals with platinum clusters, bone marrow DNA levels and superoxide dismutase activities were also recovered.

Cerium-based nanomaterials, such as ceria (CeO_2), possess the capability of scavenging ROS owing to their biomimetic antioxidant activity, in which the surface oxygen vacancies-induced coexistence system of Ce(IV) and Ce(III) can work as a redox couple.[270] CeO_2 NPs can scavenge ROS by reversibly binding oxygen and the conversion between valence states from Ce(III) to Ce(IV) on the surface of CeO_2 , thereby preventing cells from the radiation-induced oxidative damage.[271] Han et al. attempted to protect the tissues from RT by enhancing the catalytic antioxidant activities of CeO_2 (**Figure 1.17**).[272] Manganese ions deposited on the surface of CeO_2 nanocrystals formed strained Mn_3O_4 layers and increased the number of oxygen vacancies in the CeO_2 phase, which facilitated the redox reactions at the Mn_3O_4 and CeO_2 surfaces and enhanced the catalytic antioxidant activities. Only a small dose of the $\text{CeO}_2/\text{Mn}_3\text{O}_4$ nanocrystals was enough to effectively improve the survival rate of mice after RT, demonstrating that the surface strain tuning of CeO_2 nanocrystals can dramatically enhance their antioxidant properties and avoid the irradiation-induced ROS damage.

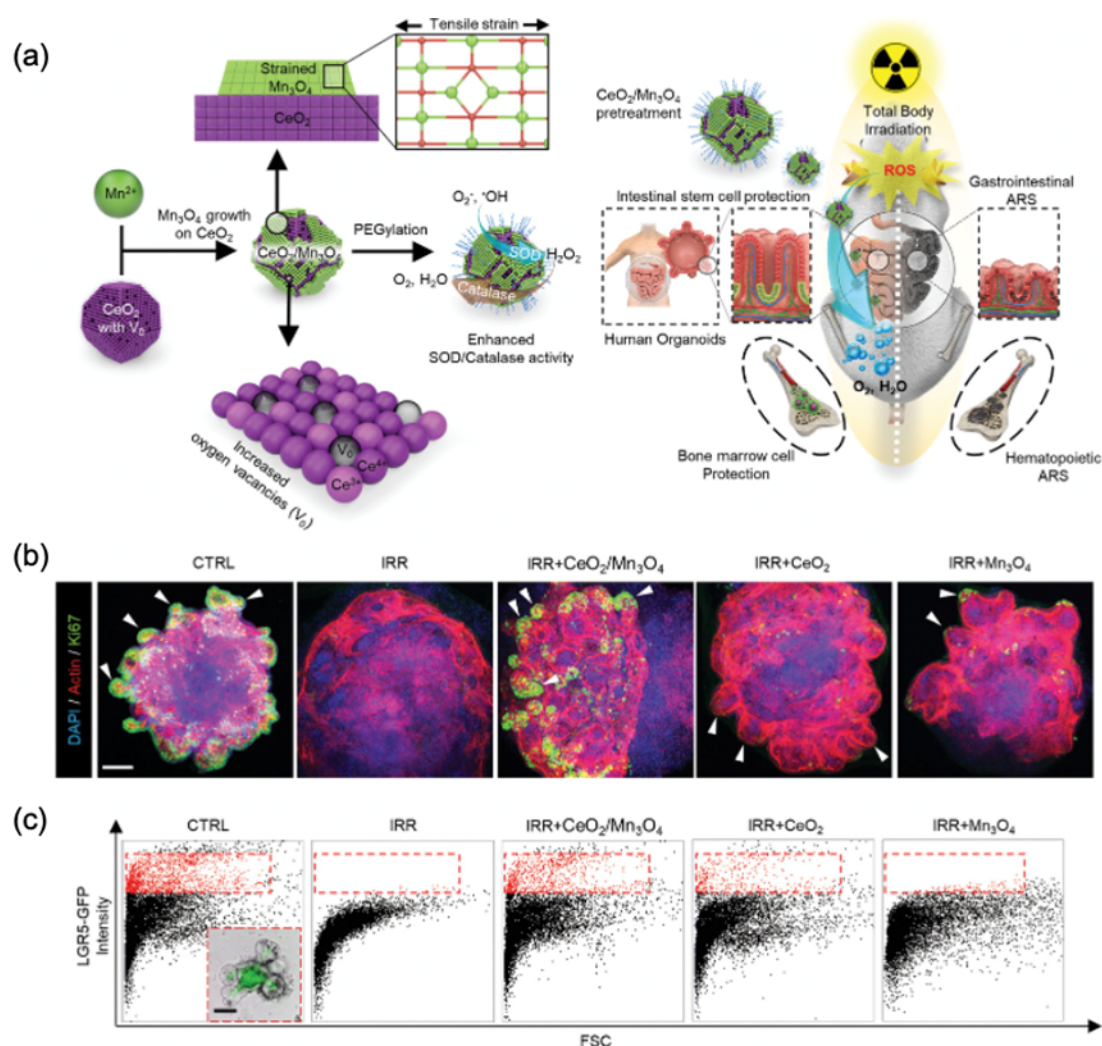


Figure 1.17. (a) Schematic illustration of highly catalytic $\text{CeO}_2/\text{Mn}_3\text{O}_4$ nanocrystals preventing acute radiation syndrome. (b) Confocal images of cells stained with Ki67, DAPI, and actin. Scale bar = 200 μm . The white arrow heads indicated representative crypt buds, signals of radioprotective effects. (c) Fluorescence-activated cell sorting analysis of dissociated LGR5-GFP mIOs 48 h after irradiation (scale bar = 50 μm).

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1.3.6. Combination Therapies

Combination therapy is a treatment modality that combines two or more therapies. Metal nanomaterials-mediated synergistic RT in combination with other therapies is becoming a promising

option in cancer treatment due to their great potential for enhancing therapeutic efficacy, inhibiting metastatic tumor growth, and improving the survival rate of cancer patients.[273] Chemoradiotherapy has emerged as an essential part of the standard care for patients with solid tumors and an effective treatment modality for metastatic tumors that are unsuitable for surgery.[274] While chemoradiotherapy improves anticancer efficacy, it also has the possibility of causing much higher toxicities than a single therapy. The side effects of chemoradiotherapy might be reduced through the improved targeting capability and controlled or stimuli-responsive drug release. Li et al. designed a pH-responsive gold nanoassembly (gold NP(RTX)) for chemoradiotherapy by linking raltitrexed (RTX, targeting and chemotherapeutic effects) to the surface of gold NPs (6 nm).[275] Gold NP(RTX) could stay assembled in the bloodstream, and most RTXs were hidden inside the nanoassembly. The nanoassembly would then disassemble and split into 6-nm gold NPs in an acid TME (pH = 6.8) to improve their penetration through the increased diffusion. The well-shielded RTXs exposed to tumor cells with the disassembly of gold NP(RTX), and the chemotherapy was thus improved due to the target delivery and controlled release of RTXs. In another work, DOX release was triggered by NIR illumination to maximize their therapeutic effect while minimizing possible side effects.[276] The hollow gold NPs designed for radiosensitization and DOX loading can also be used for the photothermal conversion under NIR irradiation. As a result of the local temperature elevation via the absorption of NIR light, increased protonation of amino groups on DOX would induce the release of DOX from the HGNPs and subsequent diffusion within tumors.

Combining thermal therapy (or hyperthermia) with RT, known as thermoradiotherapy, produces effective local control of solid tumors with manageable side effects. Hyperthermia via photothermal therapy or magnetic hyperthermia improves anticancer treatment by (a) activating and recruiting cytotoxic immune cells to tumor regions, (b) enhancing tumor cell perfusion to increase drug delivery, (c) increasing oxygen levels to improve radiation efficacy, and (d) impairing the repair of damaged DNA in cancer cells.[277] Gold nanomaterials are widely explored for photothermal therapy due to

their strong absorption in the NIR region and high heat conversion efficacy. Li et al. fabricated Arg-Gly-Asp peptides-conjugated gold nanorods (RGD-gold NRs) for synergistic thermoradiotherapy.[278] RGD-gold NRs showed a photothermal behavior with a NIR irradiation. The photothermal property enhanced the radiosensitivity in tumor cells, which further promoted the synergistic effect of RT and photothermal therapy. It is also of interest that there was a significant accumulation of cells in the G2/M phase and a drastic decrease of S phase cells after the treatment with RGD-gold NRs and NIR, suggesting that hyperthermia can also sensitize cells to radiation by regulating the cell cycle. As another high-Z element with strong absorption in the NIR region, bismuth is also a promising photosensitizer for thermoradiotherapy. Particularly, bismuth exhibits broad and strong absorption characteristics in the second near-infrared (NIR-II) window (1000-1350 nm), which allows deeper penetration and higher maximum permissible exposure.[279] Yu et al. reported the synthesis of peptide-labeled bismuth for photothermal conversion and radiosensitization.[280] Bismuth NPs were discovered to have a broad absorption range from ultraviolet to NIR. The high photothermal conversion efficiency and photostability of bismuth NPs under 1064-nm laser irradiation ensured a strong synergistic effect on tumor-bearing mice, demonstrating the promise of bismuth nanomaterials for photothermal therapy and their positive effects on RT. Magnetic hyperthermia therapy has also been introduced clinically as an alternative approach for the treatment of cancers and the enhancement of RT through heating tumor cells,[281] where the heat is generated by magnetic nanomaterials under an external alternating magnetic field (AMF).[282, 283]

Photodynamic therapy is also a non-invasive treatment modality that can be combined with RT to achieve a complementary and synergistic effect. In general, two components are essential to photodynamic therapy: photosensitizer and light source. Photodynamic therapy involves the irradiation of the photosensitizer with a light source followed by the light absorption and the generation of ROS, particularly singlet oxygen ($^1\text{O}_2$). In Oliveira and Guidelli's work, core-shell NPs

(silver@Ant NPs) consisting of a silver core and an anthracene shell were employed to increase the interaction of X-rays with the organic scintillator for ROS generation.[284] Silver@Ant NPs could trap the generated $^1\text{O}_2$ during X-ray irradiation and subsequently afford a sustained release of the trapped $^1\text{O}_2$ for up to 12 days after irradiation, which extended the RT after the irradiation sessions and led to radiation dose enhancement around silver@Ant NPs. Xu et al. developed hyaluronic acid-modified gold nanocages (gold NCs-HA) as photosensitizers. The potentials of gold NCs-HA as radiosensitizers for RT and photosensitizers for photodynamic therapy were explored simultaneously.[285] The combination of RT and photodynamic therapy resulted in a dramatic improvement in tumor elimination and effective inhibition of tumor growth compared to either therapy alone.

In order to improve therapeutic efficacy while lowering the minimum effective dose (MED) and the risks of potential side effects, other combination therapies based on metal nanomaterials, including chemodynamic/RT (**Figure 1.18**),[286, 287] immunoradiotherapy,[288] gene therapy/RT,[289] and even trimodal therapy,[290-292] have also been designed and realized. Metal nanomaterial-mediated combination therapies will certainly further inspire the field and provide novel and effective solutions for clinical anticancer treatments.

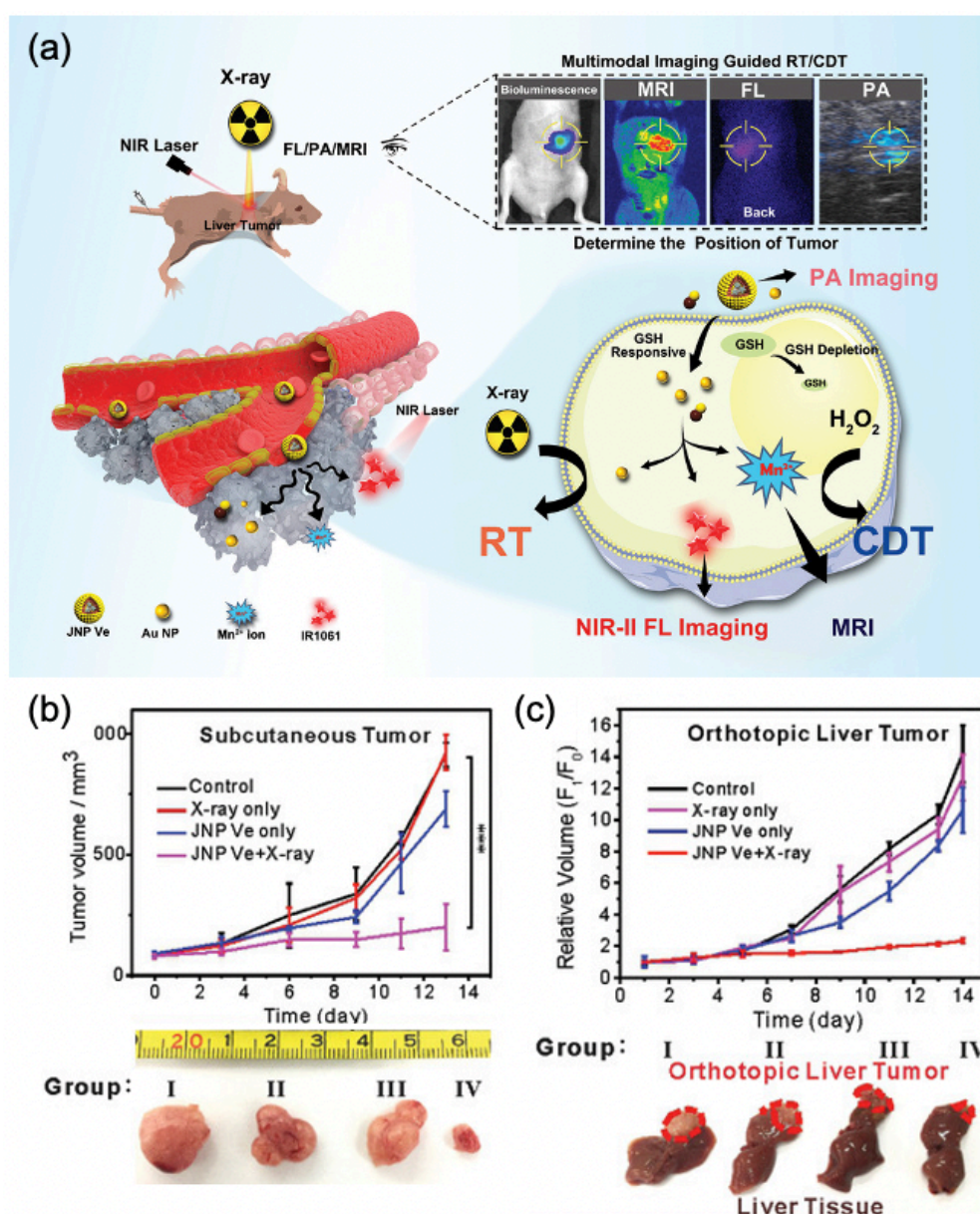


Figure 1.18. (a) Schematic illustration of image-guided RT against deep-seated tumors. GSH-induced disassembly into small gold NPs, Mn²⁺, and IR1061 penetrate into the deeper tumor tissues for RT/CDT. (b) Time-dependent relative subcutaneous tumor volume growth curves and photographs of subcutaneous tumor tissues *ex vivo* (Group I-IV represent control, X-ray only, JNP Ve only, and JNP Ve+X-ray, respectively) (c) Time-dependent relative orthotopic liver tumor volume growth curves and photographs of orthotopic liver tumor tissues and liver tissues *ex vivo*.

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1.4. Summary and Prospects

The clinic needs to maximize therapeutic effect and minimize the impact of side effects are driving advances and innovations in anticancer treatments. Due to their attractive properties, including safety, the potential for cost-effective and large-scale synthesis, ease of modifications, and a variety of structures and functions, metal nanomaterials have been widely used as radiosensitizers, imaging agents, and delivery vehicles, radioprotectors, or therapeutic agents for the RT enhancement. The size, shape, surface modification and functionalization, and administration route of metal nanomaterials can be designed to regulate their *in vivo* behaviors, including metabolism, biodistribution, toxicity, injectability, as well as their *in vivo* performance. Consequently, prolonged circulation, maximum accumulation at target lesion, and minimal distribution in neighboring healthy tissue are enabled, which ultimately optimizes and maximizes the functions of the predesigned metal nanomaterials. However, there is no doubt that a certain number of challenges should be overcome to translate these strategies into clinical applications for RT enhancement:

(a) Cost and quality control during scale-up synthesis of metal nanomaterials. Many factors must be considered in the process of scaling up from laboratory to production, such as the possible impact on formulations when synthesizing metal nanomaterials from milligram to gram or even kilogram quantities. The stability of the scale-up production process and the availability of raw materials should also be considered. In order to manage the complexities and control the cost and quality of synthesized metal nanomaterials, key steps during scale-up synthesis need to be monitored and evaluated.

(b) Systematical evaluations of *in vivo* biocompatibility, acute and long-term toxicity, pharmacokinetics, biodistribution, and metabolism of metal nanomaterials. The toxicity and biodistribution of metal nanomaterials have been extensively studied, but the majority of these assessments are short-term. In particular, the current assessment may be somewhat different from the practical applications and may not be entirely clinically applicable. Hence, it is essential to

systematically study the long-term behavior and performance of metal nanomaterials for the guidance of their clinical applications.

(c) Ready-to-use devices or kits for end users. Although metal nanomaterials have been found effective in RT enhancement, the complicated manufacture and use might not be surgeons-friendly. As a result, ready-to-use devices or kits based on these metal nanomaterials are required.

(d) Long-term stability. Maintaining the designed function throughout the life cycle is critical to the ultimate therapeutic outcome of the synthesized metal nanomaterials. Exploring strategies to maintain the stability of metal nanomaterials during long-term storage or transport is important, as is establishing stability criteria.

(e) Clinical translation of metal nanomaterials. Numerous studies have explored the applications of metal nanomaterials for RT enhancement, yet very few have reached the clinical translation stage. Five major challenges need to be considered when it comes to promoting the clinical translation of nanomedicines,[293] i.e., (1) commercial and practical feasibility, (2) clinical development feasibility, (3) translating preclinical efficacy to clinical outcome, (4) bridging preclinical toxicology to patient safety, and as discussed, (5) chemistry, manufacturing, and control. Moreover, the collaboration between researchers, engineers, surgeons, patients, and manufacturers is also essential for clinical translation.

In conclusion, enhanced RT incorporated with metal nanomaterials is a promising and burgeoning field. Although the challenges still exist in the field, metal nanomaterial-based applications are expected to continue providing novel and reliable concepts, tools, and strategies for resolving medical and surgical problems in RT enhancement. Researchers, engineers, and manufacturers can control and enhance the structure and functions of metal nanomaterials at the design and manufacturing stage, and feedback from surgeons and patients can further optimize metal nanomaterial-based nanomedicines, which will ultimately ensure the applications of metal nanomaterials in clinical RT enhancement in diverse ways and maximize therapeutic outcomes.

1.5. Objectives of This Study

Metal-based nanomaterials play essential roles in RT enhancements. For example, gold-based fiducial markers are integral to IGRT. By identifying the fiducial marker, IGRT can accurately track the position of a tumor to avoid geometric uncertainties caused by body motions during RT. IGRT can therefore improve the precision of radiation delivery and reduce the doses to the surrounding tissues. However, currently used fiducial markers with large physical dimensions would lead to complicated and invasive insertion procedures, while injectable fiducial markers based on smaller-sized gold nanoparticles (Au NPs) are obviously promising and preferred in IGRT to relieve patients' pain and improve their clinical experience. Therefore, this study aims to synthesize Au NPs via green, effective, and biocompatible methods for fiducial marker applications to achieve optimal therapeutic efficacy and minimal treatment toxicity, including:

- (a) Synthesizing Au NPs using the microwave-induced plasma-in-liquid process (MWPLP) with the addition of alginate for long-term storage of Au NPs;
- (b) Synthesizing Au NPs via a green ethanol reduction method with the addition of alginate to improve the reducing capacity of the ethanol system and the stability of the synthesized Au NPs;
- (c) Evaluating the potentials of Au NPs as injectable and minimally invasive fiducial markers based on their injectability and imaging performance;
- (d) Developing a ready-to-use injectable fiducial marker based on the synthesized Au NPs and a body temperature-activated *in situ* gel-forming system to ensure its long-term retaining *in vivo*, as well as to avoid its movement or Au NPs leakage *in vivo*.

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Chapter 2. Alginate-Stabilized Gold Nanoparticles Prepared Using the Microwave-Induced Plasma-in-Liquid Process with Long-Term Storage Stability for Potential Biomedical Applications

Abstract

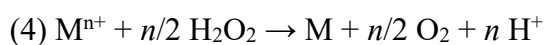
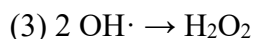
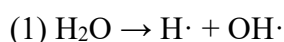
A one-step synthesis of alginate-stabilized gold nanoparticles (alg-Au NPs) using the microwave-induced plasma-in-liquid process (MWPLP) was reported in this chapter. Effects of alginate with various concentrations on the synthesis and properties of the synthesized Au NPs, including reaction rate, morphology, size, and optical absorption property, were studied. The introduction of alginate (1) accelerated the reaction rate, (2) prevented aggregation and deposition due to long time discharge in MWPLP, and (3) provided long-term colloidal stability. An abnormal size change (from large to small) of Au NPs during particle growth, which was opposite to the typical change in bottom-up chemical reduction, was observed and a possible mechanism was proposed based on the dynamical and thermodynamical instability of particles during growth. The strategy of drying and redispersion of Au NPs in alginate solution was also studied. The drying and redispersion process had an imperceptible effect on the Au NPs. Consequently, this strategy might be an effective technique for the long-term storage of Au NPs. The alg-Au NPs without the addition of toxic reducing or stabilizing agents can be appropriate to biomedical applications.

2.1. Introduction

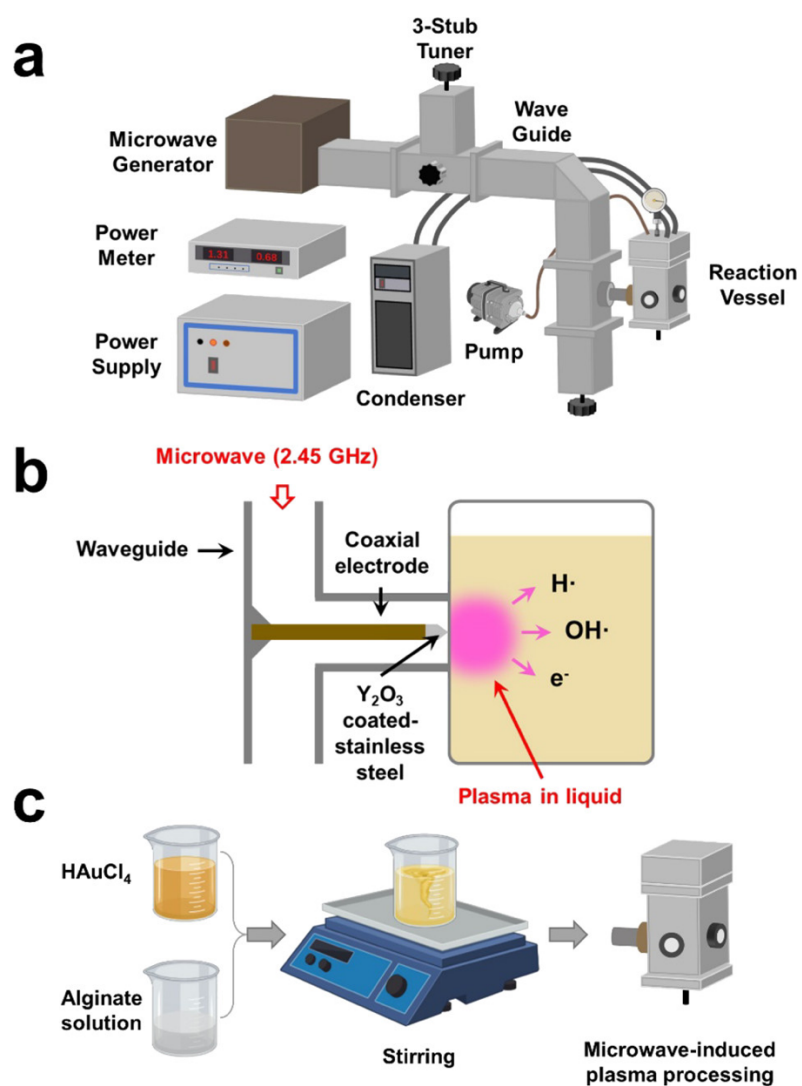
Gold nanoparticles (Au NPs) are one of the most investigated nanomaterials that exhibit great potential in various biomedical applications, such as imaging, therapy, immunotherapy, drug delivery system, etc.[1] For example, the property of inducing a strong X-ray attenuation of gold makes Au NPs an ideal candidate for CT contrast agents.[2-4] The function design, such as surface modification with peptide,[5] enables the selective and sensitive targeting of the tumor while inducing a distinct contrast in CT imaging (increased X-ray attenuation).[6] Au NPs can also be imaged via terahertz microscopy by converting NIR light into heat.[7] Regarding therapeutic applications, Au NPs show great promise as photothermal therapeutic agents. This is because the surface plasmon resonance (SPR) of Au NPs can be designed by tailoring their size, shape, composition, and structure to absorb light in the NIR range in which minimum absorption by biological molecules, such as hemoglobin, and water is observed.[8] For drug delivery purposes, Au NPs could be conjugated to a variety of antitumor substances by simple physical adsorption or by using alkanethiol linkers.[9] For building a delivery system, target molecules (e.g., cetuximab) can be applied along with the active substance to ensure better anchoring and penetration of the complex into the target cells.

Of all the methods for synthesizing Au NPs, chemical reduction is the most used strategy in which gold ions in a precursor are reduced by reducing agents such as ethanol,[10, 11] polyol,[12] sodium borohydride,[13, 14] β -d-glucose,[15] glycerol,[16] sodium citrate,[17]

etc. On the other hand, to prevent the aggregation and flocculation of Au NPs in liquid, stabilizing agents such as poly(vinyl alcohol) or poly(vinyl pyrrolidone) are also used.[10, 11, 18] However, byproducts might be generated during reactions due to the introduction of reducing and stabilizing agents. These agents may also bring biological toxicity, and therefore, washing and water treatment are indispensable. In contrast, the plasma-in-liquid process represents a green alternative in NP synthesis.[18-25] Especially, the microwave-induced plasma-in-liquid (MWPLP) process is very interesting due to its relatively low energy consumption and no need for using toxic reducing and stabilizing agents (**Scheme 2.1a**).[18, 22, 26] In MWPLP, plasma is created in three stages: (1) bubble formation, in which the liquid surrounding the electrode is heated by joule heating via microwaves, resulting in the outburst of bubbles;[27] (2) initiation of plasma, in which plasma is initiated toward the gas (provided by bubbles)/liquid interface; (3) formation of the plasma in liquid.[28] MWPLP is constituted based on a gas phase in a liquid phase. In the MWPLP apparatus (**Scheme 2.1b**), the plasma generated under low pressure can decompose water molecules to form highly reactive species, including hydrated electrons and hydrogen and hydroxyl radicals. Reaction equations for this process are summarized in eqs 1-4:



In these equations, M, H $\cdot$, and OH $\cdot$ represent a metal, hydrogen radicals, and hydroxyl radicals, respectively. Water is decomposed by plasma to form hydrogen and hydroxyl radicals (eq 1). Then, hydrogen radicals reduce metal ions (eq 2). H₂O₂ generated based on the combination of hydroxyl radicals also reduces metal ions (eqs 3 and 4).[21] Although MWPLP can be used for the green synthesis of Au NPs, the challenges of the low reaction rate, excessive reaction-induced aggregation, and long-term stability of Au NPs still remain for further biomedical applications. Sodium alginate is a natural polymer with excellent biocompatibility, low toxicity, and mild gelation ability in divalent cation buffer. Alginate molecules can be dissolved in water because of negatively charged carboxyl groups. For an accelerated reaction and better stability, alginate was introduced in MWPLP (**Scheme 2.1c**). In this chapter, the effects of sodium alginate on the synthesis, morphology, size, and optical absorption property of Au NPs were studied. The proposed mechanism for the formation of Au NPs in alginate via MWPLP was demonstrated. Also, the strategy of drying and redispersion of Au NPs in alginate solution was studied and presented for the long-term storage of Au NPs and other metal NPs.



Scheme 2.1. (a) Experimental setup of MWPLP for the synthesis of Au NPs. (b) Schematic illustration of microwave-induced plasma in the reaction vessel. (c) Fabrication route for synthesizing Au NPs in aqueous solutions of sodium alginate and AuCl_4^-

2.2. Experimental Section

2.2.1. Materials

Tetrachloroauric (III) acid hydrate ($\text{HAuCl}_4 \cdot n\text{H}_2\text{O}$, $n=3.7$, Kojima, Japan) and sodium alginate (I-1 (molecular weight: 1.5×10^6) and I-8 (molecular weight: 3.2×10^6), Kimica Corp., Japan) were used as a precursor and an additive, respectively. All “alginate” described in this chapter without indication is sodium alginate I-1. 1g of $\text{HAuCl}_4 \cdot n\text{H}_2\text{O}$ ($n = 3.7$) was dissolved in 100 mL of pure water to obtain 24.6 mM aqueous HAuCl_4 . All chemicals were used as received. Pure water (Organo/ELGA Purelabo system, $>18.2 \text{ M}\Omega \cdot \text{cm}$) was used to prepare solutions for plasma reaction.

2.2.2. Apparatus

A schematic illustration of the homemade microwave-induced plasma apparatus with a reactor is shown in **Scheme 2.1(a)**. Microwave (2.45 GHz) was emitted from a magnetron (Micro Denshi UW-1500) and passed through a power meter, a tuner, a WRJ-2 rectangular waveguide ($109.22 \times 54.61 \text{ mm}$) to coaxial adaptor, and a coaxial plasma source electrode. The coaxial electrode was attached in the middle of the waveguide, and its front was projected into the chamber. This electrode terminated with an Y_2O_3 -coated stainless-steel tip. Contaminations from the electrode were suppressed using this ceramic-coated electrode, as this electrode material does not dissolve in the reaction solution during plasma ignition. The inside of a stainless-steel reactor (500 cm^3) was coated with PTFE. The plasma reaction

solution was cooled using a stainless-steel cooling spiral containing chilled liquid at 0 °C. The reaction temperature was measured with a thermocouple. When the microwave output was fixed at 500 W, the reaction temperature was increased by the plasma irradiation, reaching 33 °C after 3 min, and then remained at 33 °C for the duration of the irradiation. Plasma ignition and the solution during the plasma reaction can be observed by eye through a quartz window. The pressure was decreased using a diaphragm vacuum pump and measured with a vacuum gauge.

2.2.3. Synthesis of Gold Nanoparticles

The synthesis route is illustrated in **Scheme 2.1(c)**. 0.5, 1.0, and 2.0 % w/v aqueous sodium alginate solutions were prepared by dissolving 0.5, 1.0, 2.0 g sodium alginate into 100 mL of water, respectively. 4.08 mL aqueous HAuCl₄ solution (24.6 mM) was added into 195.92 mL of water to obtain HAuCl₄ solution of 0.5 mM. The volume ratio of HAuCl₄ and alginate solution was 2:1. The mixed solution was stirred for 3 h for getting homogeneous solutions and then introduced into the reaction vessel. The synthesized Au NPs were labeled with sodium alginate concentration: 0.5, 1.0, and 2.0 % w/v. The microwave output was kept at 500 W during the reaction. The reaction lasted for 10, 20, 30, 40, 50, 60 min for pure water group (Au NPs synthesized in pure water) and 10, 15, 20, 25 min for sodium alginate groups (Au NPs synthesized in alginate solutions).

2.2.4. Drying and Redispersion of alg-Au NPs

After synthesis, alg-Au NPs suspensions were concentrated by a rotary evaporator (Eyela NVC-2100, Tokyo Rikikai Co. Ltd.). Then the concentrated alg-Au NPs suspensions were collected and transferred to a vacuum oven for further drying. For the redispersion of alg-Au NPs, alg-Au NPs were weighed and dispersed in DI water to the original concentrations.

2.2.5. Characterizations

UV-Vis extinction spectra of the obtained Au NP dispersions were collected to observe Au NPs formation at different time by using a UV-Vis spectrophotometer (Shimadzu UV-1800) and a quartz cell with 1 cm optical path. During the plasma reaction, 3 mL of the sample solution was taken directly from the reaction vessel for each measurement at various reaction times. Transmission electron microscopy (TEM, JEOL JEM 2000-ES, at 200 kV) with an energy dispersive X-ray spectroscopy (EDS) was used to analyze the morphology, size, elemental composition, and Au NPs. The X-ray diffraction (XRD) patterns were collected by using a Rigaku Miniflex II X-ray diffractometer. For TEM sample preparation, Au NPs dispersion was dropped on collodion film-coated copper TEM grids and left for naturally dried. The particle size and size distribution of more than 100 Au NPs were measured from TEM images by Image-J (available as freeware from <http://rsbweb.nih.gov/ij/>). Concentrations of Au NPs were determined by Inductively Coupled Plasma (ICP-AES) using

ICPE-9000 (Shimadzu) spectrometer. Zeta potentials of Au NPs dispersions were measured by the Zeta potential & particle size analyzer ALSZ-2 (Photal Otsuka Electronics, Japan). pH values were collected by pH meter D-55 (Horiba, Japan).

2.3. Results and Discussion

2.3.1. UV-Vis Spectra of the Obtained Au NPs-Alginate Aqueous Dispersions

Figure 2.1 shows the UV-Vis extinction spectra of Au NPs prepared in pure water and sodium alginate aqueous solutions of different concentrations for various reaction times. The spectra of Au NPs prepared in pure water exhibited a surface plasmon resonance (SPR) peak with a maximum absorption wavelength (λ_{max}) at around 557 nm. The λ_{max} values of Au NPs prepared in alginate solution with concentrations of 0.5, 1.0, and 2.0% w/v were at 539, 554, and 573 nm, respectively. The peak intensities of these SPR peaks of Au NPs prepared in the solutions with different concentrations of sodium alginate did not change obviously, indicating that sodium alginate had no significant effect on the yield of Au NPs. The red shifts of λ_{max} (1.0 and 2.0% w/v groups) were caused by weak aggregation states of the adjacent Au NPs due to the surge in the number of Au NPs. As shown in **Figure S2.1**, after being diluted to the same sodium alginate concentration of 0.5% w/v group, the 1.0 and 2.0% w/v groups showed shorter λ_{max} (533 and 529 nm) than that of 0.5% w/v group (539 nm). For Au NPs prepared in pure water, it could be noted that the peak intensity reached a maximum at 20 min with the increase in reaction time. It suggests that the reaction from Au(III) to Au(0) would

require approximately 20 min or more to complete. For Au NPs prepared in aqueous solutions of sodium alginate with various concentrations, all the absorption intensities of Au NPs reached the maximum value within 10 min, suggesting that the reaction could be completed within 10 min. These phenomena strongly suggest that the addition of sodium alginate could accelerate the reaction rate from Au(III) to Au(0). Furthermore, the absorption intensity of Au NPs prepared in pure water gradually decreased after reaching the maximum value, which was caused by the reduced concentration of Au NPs in dispersion due to the excessive MWPLP-induced aggregation and deposition. Interestingly, the absorption intensities of Au NPs prepared in sodium alginate solutions were stable after reaching the maximum. The SPR peaks showed an ideal bell shape, which implies that negligible aggregation occurred, and the Au NPs are well dispersed in dispersions. These results suggest that, in the presence of sodium alginate, Au NPs did not aggregate and deposit due to the increase in reaction time, indicating that alginate can prevent the aggregation and deposition caused by excessive MWPLP.

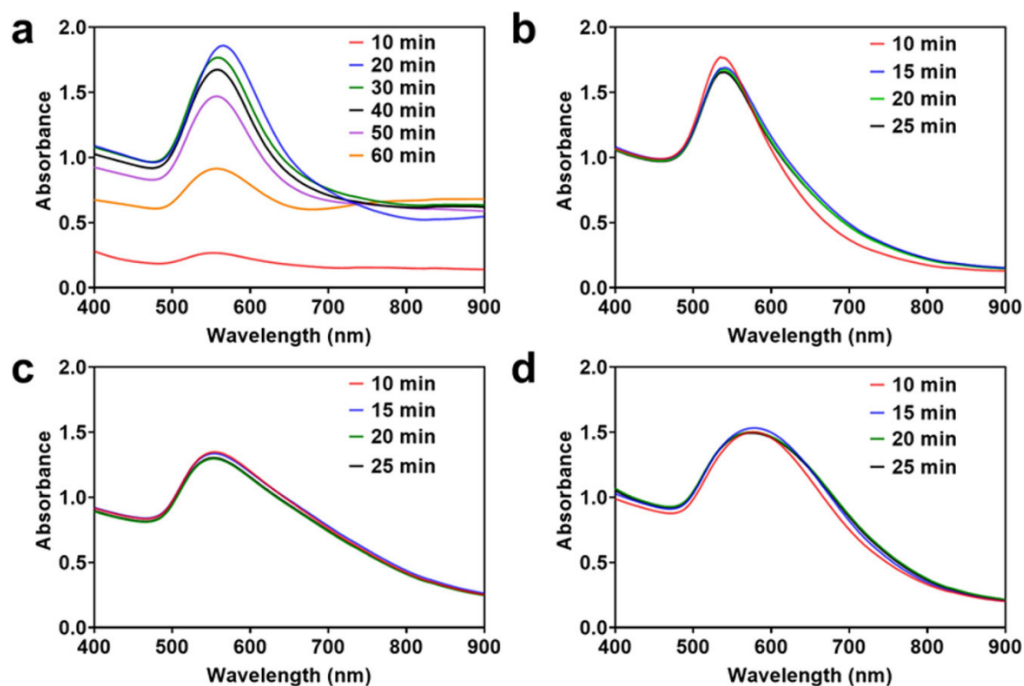


Figure 2.1. UV-Vis spectra of Au NP dispersions prepared in (a) pure water and aqueous solutions of sodium alginate with concentrations of (b) 0.5% w/v, (c) 1.0% w/v, and (d) 2.0% w/v for various reaction times.

2.3.2. Effect of the Concentration of Sodium Alginate on the Morphology, Size, and Size Distribution of Au NPs

Transmission electron microscopy (TEM) images of Au NPs prepared in pure water and aqueous solutions of sodium alginate with different concentrations (reaction time: 20 min), along with their size distributions, are collected in **Figure 2.2a** and **Figure S2.2**. As shown in **Figure 2.2a**, the Au NPs prepared in pure water are mostly spherical, while a few show triangular and hexagonal shapes. It was previously reported that triangular and hexagonal particles were obtained from the synthesis of Au NPs using the plasma-in-liquid process.[29] When they occurred on the surface of Au-nucleated seeds, reductions were dependent on the

surface energy of the different crystal facets, and polygonal particles were generated.[18] In contrast, almost all the Au NPs prepared in sodium alginate solution showed spherical shapes. In addition, the Au NPs prepared in pure water contacted each other and showed a tendency to aggregate because these particles were naked (without coating of sodium alginate). After MWPLP in sodium alginate solutions, Au NPs were uniformly dispersed. **Figure S2.3** shows the zeta potentials and pH values of Au NP dispersions. Zeta potentials of Au NP dispersions prepared in alginate (-40.60 to -55.17 mV) are more negative than that prepared in pure water (-10.80 mV). Therefore, the electrostatic repulsion of Au NPs prepared in pure water is weaker than the electrostatic and steric repulsion provided by -COO^- groups of alginate chains.[30, 31] Accordingly, the Au NPs prepared in pure water are more easily aggregated than that prepared in the presence of alginate. In **Figure 2.2b**, starting from 10 min, the particle size of the Au NPs prepared in pure water increased with increasing reaction time and reached a relatively stable value (around 68 nm) within 30 min. This is a result of the assembly and merging between Au NPs due to the lack of capping agents.[32, 33] As the reaction time increased further, although the size stopped increasing, Au NPs started to aggregate and deposit, as shown in **Figure S2.4**. Conversely, as illustrated in **Scheme 2.2**, after the addition of alginate, the chelation between gold chloride complexes and carboxyl and hydroxyl groups would allow gold ions to evenly disperse in solution.[34] After MWPLP, Au NPs could be generated in the alginate chain-formed cavities. The formed Au NPs anchored strongly with the functional groups (-COO^- and -OH) of alginate. This helped

prevent further assembly, merging, and aggregation of Au NPs.[30] The sizes of Au NPs were almost constant at different reaction times from 10 to 25 min, indicating that the reactions could be completed within 10 min with the assistance of alginate. At the same time, no aggregation and deposition are observed in **Figure S2.5**, reflecting that the addition of alginate could indeed accelerate the reaction and prevent subsequent aggregation and deposition caused by inordinate MWPLP. By comparing the sizes and size distributions of Au NPs produced in different concentrations of alginate solution in **Figure 2.2c**, the size of Au NPs gradually decreased with increasing alginate concentration. This is because alginate forms cavities and functions as a template for the growth of the Au NPs.[31] The alginate chains closer to each other in a higher concentration of alginate solution would form smaller cavities that could limit the uncontrollable growth of Au NPs. The size of Au NPs has a significant impact on their biomedical applications. For example, for all Au NPs with sizes ranging from 10 to 250 nm, the majority of gold was present in the liver and spleen.[35] A clear difference could be observed between the distribution of the 10 nm particles and the larger particles. The 10 nm particles are present in various organ systems including blood, liver, spleen, kidney, testis, thymus, heart, lung, and brain, whereas the larger particles can only be detected in blood, liver, and spleen. In addition, the size of Au NPs also affects their metabolism,[36] cytotoxicity,[37] and radiosensitization.[38] The sizes of Au NPs changed in a large range (from 41 to 12 nm) based on the concentration control of alginate solution (from 0.5 to 2.0% w/v). Therefore, the size of Au NPs can be controlled by tuning the

concentrations of alginate solutions for broadening their applicability in a variety of biomedical applications.

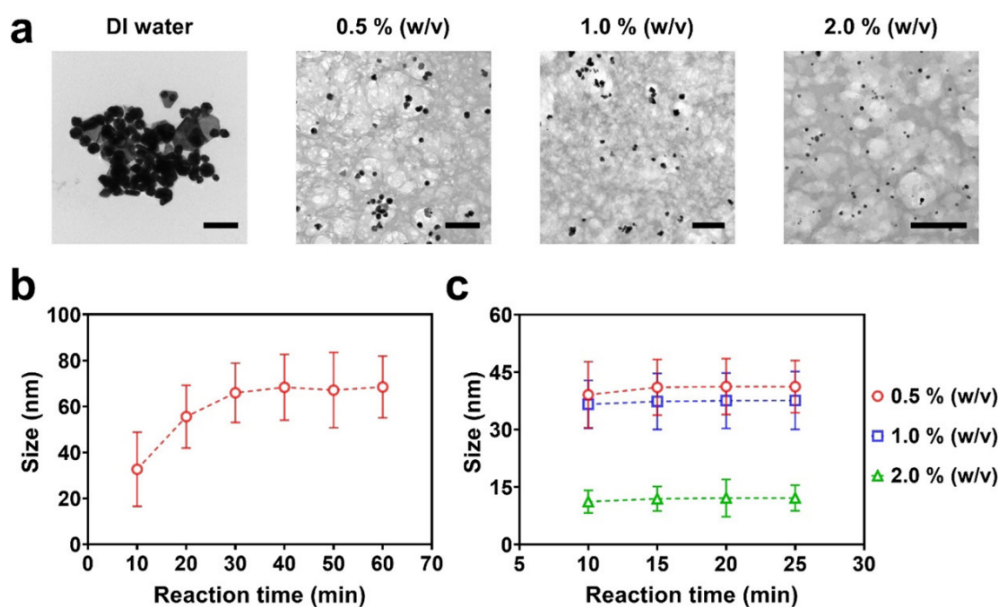
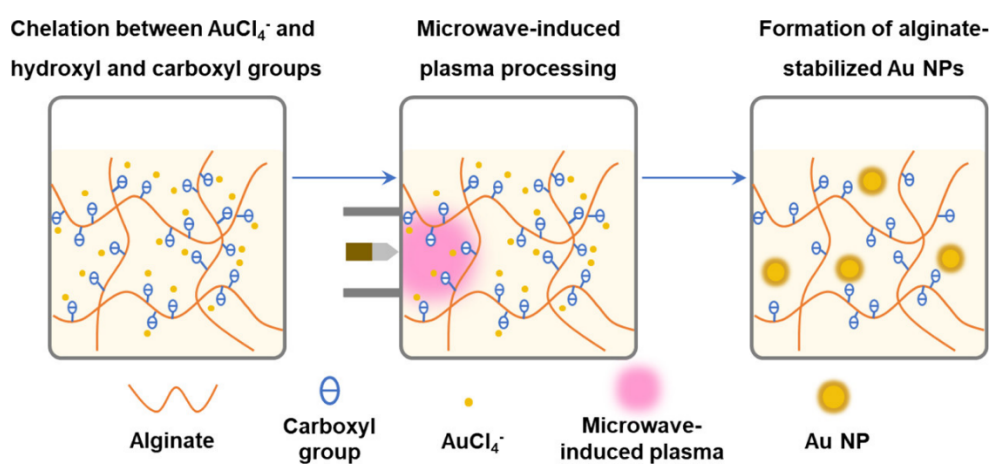


Figure 2.2. (a) TEM images of Au NPs prepared in pure water and sodium alginate aqueous solutions (reaction time: 20 min). Scale bars are 200 nm. (b) Average size of Au NPs prepared in pure water with different reaction times. (c) Average size of Au NPs prepared in alginate aqueous solution with different concentrations and reaction times.



Scheme 2.2. Illustration of the formation process of alg-Au NPs

2.3.3. Formation Mechanism of Au NPs Using MWPLP

Alginate can accelerate the reaction and all reduction reactions can complete within 10 min, as evidenced by both results of UV-Vis spectra and the sizes of Au NPs prepared in alginate solutions in comparison with that in pure water. To investigate the formation of Au NPs during the initial stage of the reaction, Au NPs were collected at 1, 2, 3, 4, and 5 min with an alginate solution concentration of 2% w/v for characterizations. **Figure 2.3a, b** shows the TEM images and average sizes of Au NPs prepared in alginate solutions (2.0% w/v) with different reaction times. It could be noted that differing from the Au NPs prepared in water with increasing size over time, the size of the Au NPs prepared in alginate solutions decreased in the first 5 min. In **Figure 2.3c**, the absorption intensity increases with reaction time, indicating that Au(III) was gradually transformed into Au(0). The blue shift of the SPR peak in the first 5 min of the reaction is a result of the breakdown of Au NPs from their aggregation as found previously (**Section 2.3.1**) and observed in TEM. Interestingly, aggregations with a diameter of about 100-150 nm were found in the first minute, as shown in **Figure 2.3d**, and disappeared within 2 min. The EDS result suggests that the aggregation was Au(0) (**Figure S2.6**). At the same time, the yield of Au NPs (**Figure 2.3e**) calculated based on ICP data was 96.82% in the first minute, which means that 96% of Au(III) transformed into Au(0). The yield decreased and stabilized at around 85% after 2 min, suggesting that about 11% of Au(0) turned back into Au(III). These results imply that Au NPs may be generated from large gold aggregations during the reaction. The XRD result (**Figure S2.7**) after 25 min reaction

supported the presence of Au. Further, the size of Au NPs was gradually reduced due to dissolution under the decreasing pH condition during this process, as shown in **Figure 2.3f**. To validate this process, a low-concentration alginate solution (1.0% w/v) was used to appropriately slow down the reaction rate. TEM images (**Figure S2.8**) show that large-size aggregations also existed in the early stage of the reaction, and EDS confirmed the component of the aggregation (**Figure S2.9**). The size of Au NPs also decreased with increasing reaction time (**Figure S2.10**). Based on the results, a schematic diagram of the proposed formation mechanism of Au NPs is shown in **Figure 2.3g**. Briefly, alginate with the assistance of high temperature generated by MWPLP shows relatively strong reducibility[30, 39] and results in a dynamical instability. The dynamical instability dramatically accelerates the transformation of Au(III) to Au(0) in a very short time. The rapid growth of Au NPs in alginate bubbles (or cavities) leads to the formation of Au aggregations. The Au aggregations are in a high-energy state because of the quick reduction process. At the same time, alginate is an anionic polymer with high charge density, and the negatively charged alginate facilitates the attraction and crack of high-energy Au aggregations for lower energy.[30] After that, the Au aggregations crack into irregular Au NPs, and the reaction will continue with the presence of microwave-induced plasma and alginate. Also, the irregular Au NPs gradually become spherical NPs for thermodynamic stability.[40] However, due to the decrease in pH and the formation of cavities by alginate chains, which can function as a template for the Au NPs, the size of the Au NPs decreased and was gradually stabilized. The formation of Au NPs may therefore be

composed of five steps: (1) rapid growth of Au NPs in alginate bubble (or cavity), (2) formation of Au aggregation due to alginate-enhanced reducibility, (3) crack of Au aggregation for lower energy and higher stability, (4) further reaction for lowering surface energy, and (5) stabilization of Au NPs.

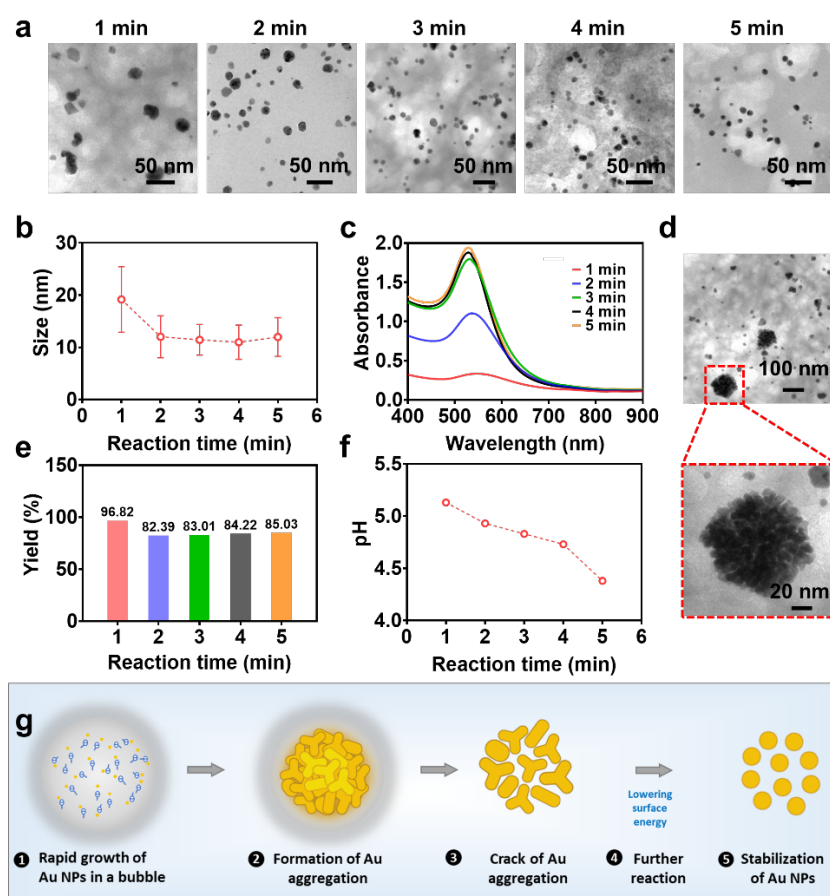


Figure 2.3. (a) TEM images of Au NPs prepared in alginate aqueous solutions (2.0% w/v) at different reaction times. Scale bars are 50 nm. (b) Average size of Au NPs prepared in alginate aqueous solutions (2.0% w/v) for different reaction times. (c) UV-Vis spectra of Au NPs prepared in alginate aqueous solutions (2.0% w/v) with different reaction times. (d) TEM images of Au aggregation with a reaction time of 1 min. (e) Yield of Au NPs prepared in alginate aqueous solutions (2.0% w/v) with different reaction times. (f) pH of Au NP dispersions with different reaction times. (g) Mechanism of Au NP formation using MWPLP.

2.3.4. Effect of Alginate on the Stability of Au NPs

Figure S2.11 shows images of Au NPs prepared in pure water on day 0, day 1, and day 7 and Au NPs prepared in alginate solutions with different concentrations on day 0 and day 60. The Au NPs prepared in water aggregated and deposited after 1 day, while a part of Au NPs prepared in alginate solutions deposited after 60 days and the rest suspended in alginate solution. Quick aggregation of Au NPs prepared in pure water is consistent with their small absolute zeta potential value (-10.80 mV, **Figure S2.3a**). Usually, NPs are considered stable if the zeta potential is more positive than +30 mV or more negative than -30 mV due to electrostatic repulsions.[25, 41] As shown in **Figure S2.3a**, all Au NPs prepared in alginate solutions have zeta potentials more negative than -30 mV, which make them stable for much longer time compared with Au NPs in pure water. The aggregation degree of metal NPs could also be effectively reflected by changes in the absorption characteristics, especially the shift of the SPR peak in the UV-Vis spectra.[42, 43] If strong aggregation occurred for Au NPs after a long storage period, a strong red shift of the SPR peak would be observed in the UV-Vis spectra.[30] As shown in **Figure 2.4**, after 60 days, the absorption intensity of Au NPs prepared in different concentrations of alginate decreased, indicating that the total amount of Au NPs in dispersions was reduced. Surprisingly, the blue shift, instead of red shift, of the SPR peak could be observed, suggesting that the size of Au NPs in the dispersions after 60 days was smaller than that of the samples on day 0. Therefore, it can be inferred that after 60 days of storage, mainly large-sized Au NPs deposited, while small-sized Au NPs were still

suspended in alginate solution. Compared with Au NPs prepared in pure water, the addition of alginate can stabilize small-sized NPs and slow down the deposition rate of large-sized Au NPs.

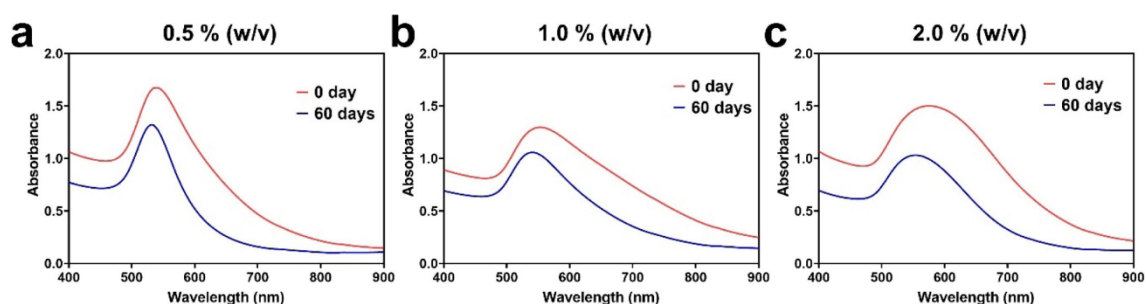


Figure 2.4. UV-Vis spectra of alg-Au NP dispersions at 0 day and 60 days after synthesis with various sodium alginate concentrations: (a) 0.5% w/v, (b) 1.0% w/v, and (c) 2.0% w/v.

2.3.5. Drying and Redispersion of Au NPs-Alginate Aqueous Dispersions

Keeping the stability of Au NPs is an important prerequisite for maintaining their designed functions and further biomedical applications. The low stability of Au NPs can complicate their use in biological environments, in particular, the formation of irreversible aggregations when they are subjected to physical and chemical changes (e.g., contact with biofluids, freeze-drying, and ion strength gradients).[44] Further, the low stability also complicates various steps of the manufacturing and shipping processes.[45] Up to now, some compounds such as surfactants[46] or cyclodextrin[47] have been used to modify NPs to counter the challenge of instability.[48] Despite the advances made with those methods, the challenge remains in producing highly stable NPs and retaining the long-term stability of

functionalized NPs.[49] The strategy of drying and redispersion was therefore adopted to maintain the stability and dispersibility of Au NPs. This is based on the cavities formed by alginate chains that could function as a template and provide barriers to prevent Au NPs from contacting each other. From **Figure 2.5**, the UV-Vis spectrum results showed that there was no significant change in the absorption intensity after drying and redissolution, indicating that the concentration of Au NPs in dispersions did not decrease substantially. But the broadening of the SPR peak implies a slight aggregation of Au NPs. By observing and analyzing TEM images as shown in **Figure 2.6a**, it can be found that the shape and dispersion of Au NPs have not changed. From **Figure 2.6b, c**, there is no significant change in the size distributions and average sizes of Au NPs before drying and after redispersion. These results reveal that the Au NPs prepared in alginate solutions could aggregate slightly, but the morphology, dispersion, and size were not significantly affected after the drying and redispersion process. In addition, the intravenously injected NPs may be rapidly cleared from the body by the reticuloendothelial system (RES) and accelerated blood clearance phenomenon (ABC phenomenon), which affect the therapeutic effect significantly. For homogeneous distribution, long-term *in situ* retaining, and low leakage, the gelation ability of Au NPs suspended in alginate solution was studied before drying and after redispersion, as shown in **Figure 2.7**. All alginate solutions with different concentrations exhibited fluid-like behavior after injection in the buffer with a physiological concentration of Ca^{2+} (1.8 mM). With the increase in concentration to 3.6 mM, alg-Au NPs rapidly transformed into gels, and the drying and

redissolution process had no effect on the gelation ability of alginate solutions with different concentrations. Therefore, the addition of alginate could not only accelerate the reaction and prevent aggregation and deposition but also improve the long-term storage stability of Au NPs and the convenience of storage or shipping process of a large amount of Au NPs compared with the Au NPs stored in liquid. The process of drying and redispersion with alginate as a stabilizing agent might also provide a new strategy for the long-term storage of other metal NPs.

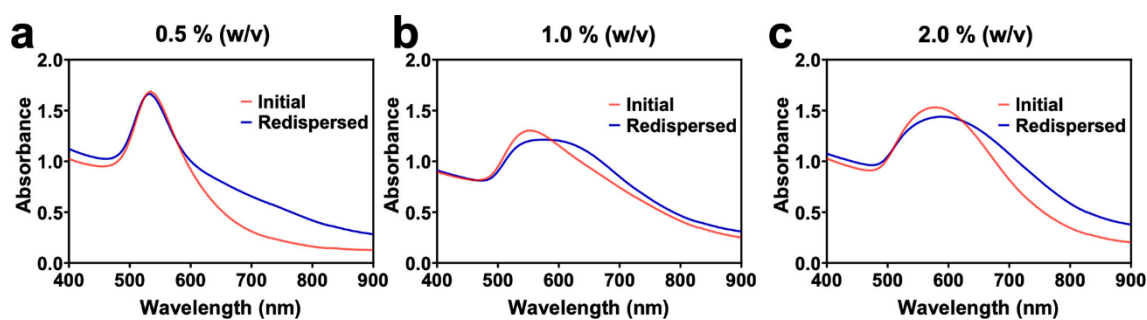


Figure 2.5. UV-Vis spectra of alg-Au NP dispersions before drying and after redispersion with various sodium alginate concentrations: (a) 0.5% w/v, (b) 1.0% w/v, and (c) 2.0% w/v.

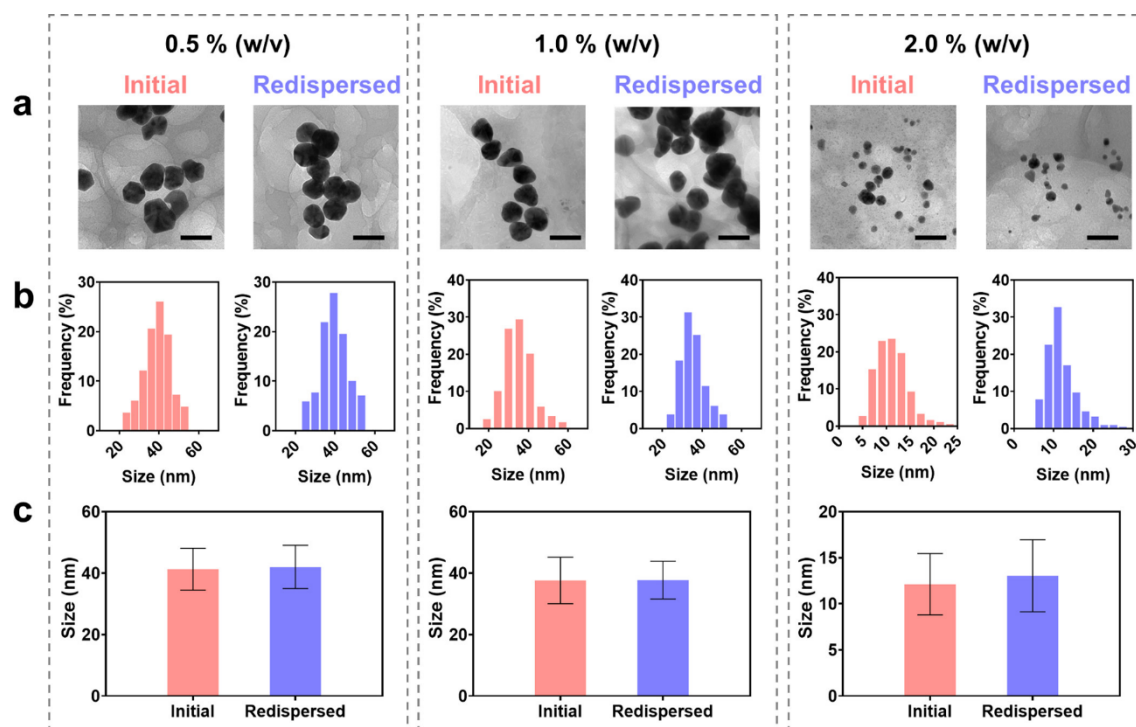


Figure 2.6. (a) TEM images, (b) size distributions, and (c) average sizes of Au NPs before drying and after redispersion with various sodium alginate concentrations. Scale bars, 50 nm.

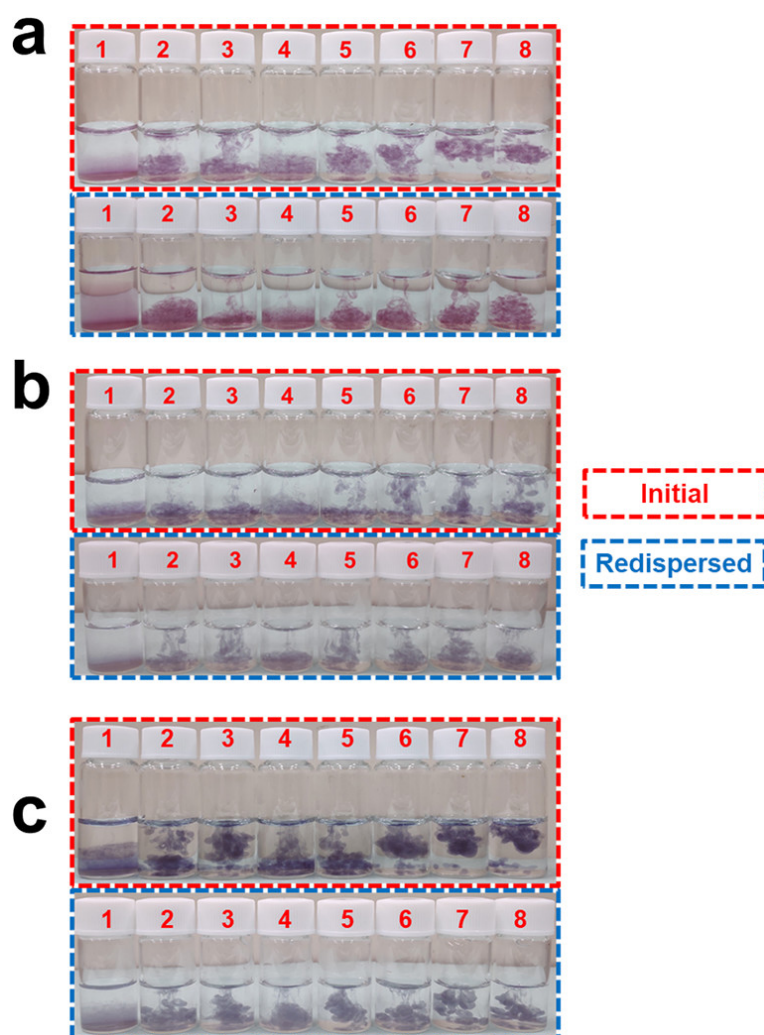


Figure 2.7. Gelation ability of sodium alginate solutions with concentrations of (a) 0.5% w/v, (b) 1.0% w/v, and (c) 2.0% w/v in Ca^{2+} buffer before drying (red) and after redispersion (blue). Ca^{2+} concentrations: #1: 1.8 mM; #2: 3.6 mM; #3: 5.4 mM; #4: 7.2 mM; #5: 9.0 mM; #6: 10.8 mM; #7: 12.6 mM; #8: 14.4 mM.

2.3.6. Accelerated Stability Test of Alg-Au NPs

As defined by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH), the accelerated stability test is designed to study the chemical degradation or physical change of a drug substance or drug product by using

exaggerated storage conditions for predicting the expiration date or lifespan under the recommended storage conditions. To estimate the long-term stability, alg-Au NPs were kept at 40 °C, which is the most commonly used accelerated stability storage condition for drug products within the USA market, for 1, 2, 3, and 4 month(s) to simulate the storage at 25 °C for 0.5, 1.0, 1.5, and 2.0 year(s). **Figure 2.8** shows the UV-Vis spectrum results of alg-Au NPs with different alginate concentrations; the negligible change in the absorption intensity with a broadening SPR peak implies that the amount of Au NPs nearly remained constant and a minor aggregation between Au NPs occurred. The shape and dispersion of alg-Au NPs also did not change after being kept for 4 months, as evidenced by the TEM images shown in **Figures S2.12-14**. **Figure 2.9** shows that the average sizes of Au NPs stabilized with various concentrations of alginate are almost the same after the accelerated stability test. In addition, after being dispersed in pure water, the alg-Au NPs display an excellent injectability and gelation ability in Ca^{2+} buffer, as **Figure 2.10** illustrates. In an extra experiment for evaluating the universality of the drying and redispersion process on the stability of alg-Au NPs after long-term storage, type I-1 alginate (molecular weight, 1.5×10^6) was substituted by type I-8 alginate with a higher molecular weight (molecular weight: 3.2×10^6). TEM (**Figures S2.15-17**) shows that the I-8 alg-Au NPs are spherical, suggesting that increasing molecular weight of alginate did not affect the morphology of Au NPs. However, smaller average sizes and narrower size distributions of Au NPs are correlated with the increase in the molecular weight of alginate (**Figure 2.2c** and **Figures S2.15-18**). After being kept for 4 months, no apparent

changes were observed in the UV-Vis spectra (**Figure S2.19**) and average sizes (**Figure S2.18**) of I-8 alg-Au NPs. The redispersed I-8 alg-Au NPs have a superb injectability and gelation ability as well (**Figure S2.20**). These results suggest the positive effect of alginate on the stability of Au NPs, ensuring their expected performance in practical applications within the designed lifespan. It is conceivable that the introduction of alginate and the subsequent drying and redispersion process could serve as a powerful tool for the long-term storage of metal NPs. The higher stability of Au NPs stabilized by I-8 alginate than that of I-1 alginate can be ascribed to the higher viscosity of I-8 alginate and its solution. This can help reduce the collision, settling, aggregation, and deposition of Au NPs during synthesis, drying, and redispersion.

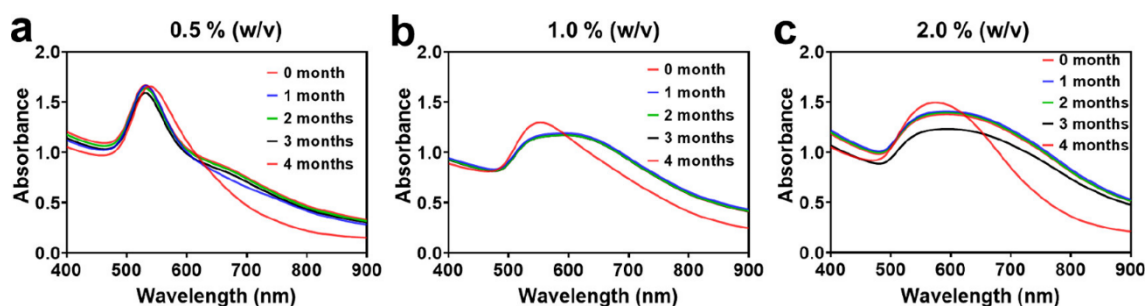


Figure 2.8. UV-Vis spectra of alg-Au NPs prepared with various sodium alginate concentrations ((a) 0.5% w/v, (b) 1.0% w/v, and (c) 2.0% w/v) after the accelerated stability test.

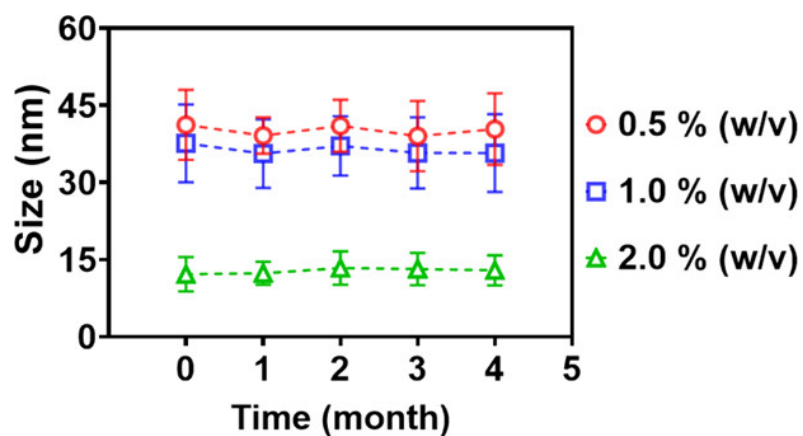


Figure 2.9. Average size of alg-Au NPs prepared with various sodium alginate concentrations after the accelerated stability test. Sodium alginate concentrations: red circle, 0.5% w/v; blue square, 1.0% w/v; green triangle, 2.0% w/v.

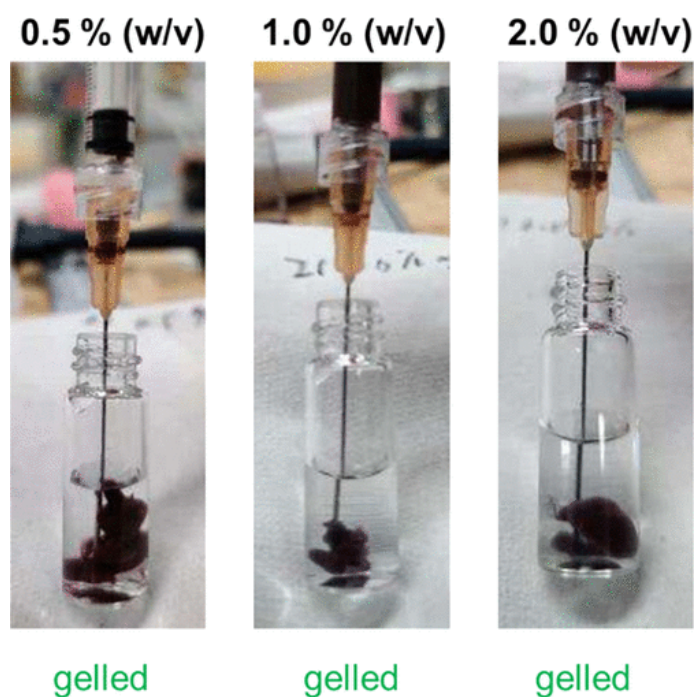


Figure 2.10. Injectability and gelation performance of alg-Au NPs in Ca^{2+} buffer (1.8 mM) after the accelerated stability test.

2.4. Conclusions

In summary, alg-Au NPs using MWPLP were prepared. The introduction of alginate could (1) accelerate the reaction rate, (2) prevent aggregation and deposition due to long time discharge in MWPLP, and (3) provide long-term stability. The size of Au NPs prepared in different concentrations of alginate ranged from 41 to 12 nm, indicating that the concentration of alginate could be used for controlling the size of Au NPs. An abnormal size change (from large to small) that is opposite to the typical particle growth in the bottom-up chemical reduction was observed. A possible mechanism of the observed phenomenon was proposed based on dynamical and thermodynamical instability. The alginate dispersions with Au NPs were dried and redispersed to evaluate the effect of the drying and redispersion process on the Au NPs. Results suggest that the process had an imperceptible effect on the Au NPs in terms of their size and dispersibility. Consequently, this strategy might be an effective technique for the long-term storage of Au NPs and other metal NPs. The stabilized Au NPs without the addition of toxic reducing or stabilizing agents can be appropriate for biomedical applications.

2.5. Supporting Information

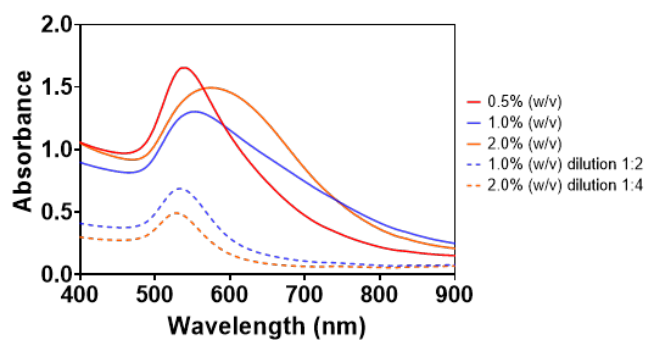


Figure S2.1. UV-Vis spectra of Au NPs suspensions prepared in aqueous solutions of sodium alginate with concentrations of 0.5, 1.0, 2.0% w/v, and their diluted solutions

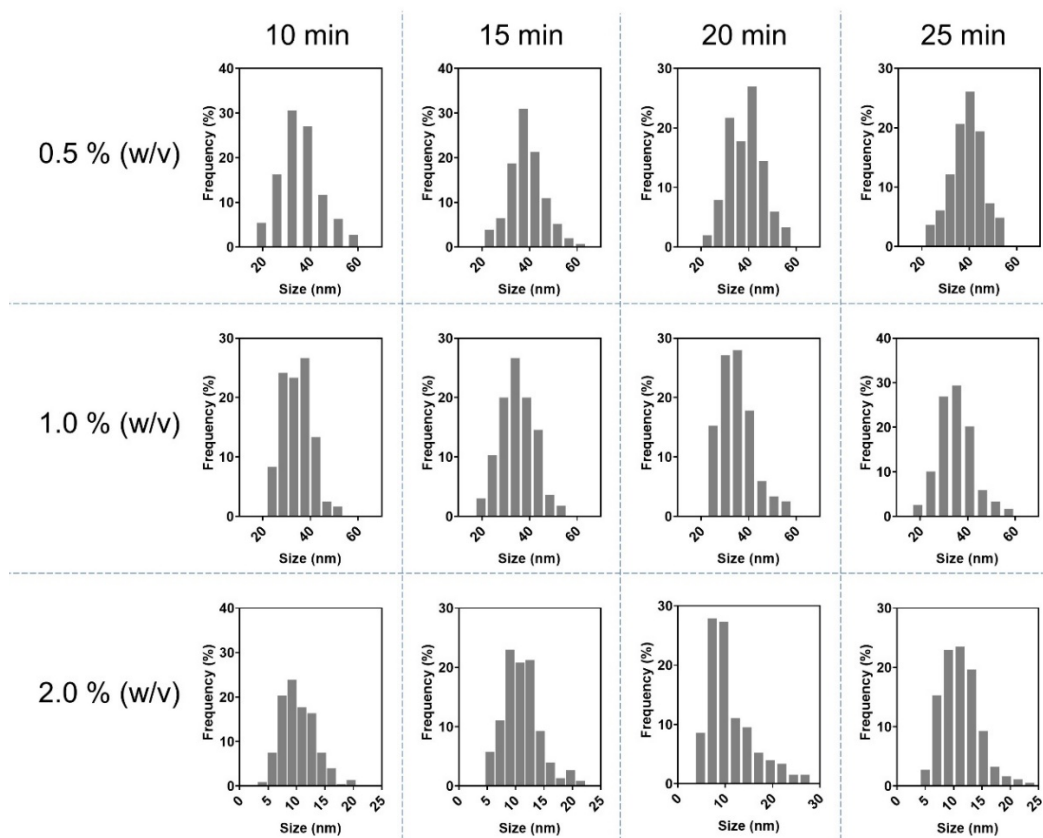


Figure S2.2. Size distributions Au NPs prepared in alginate with different concentrations and reaction times

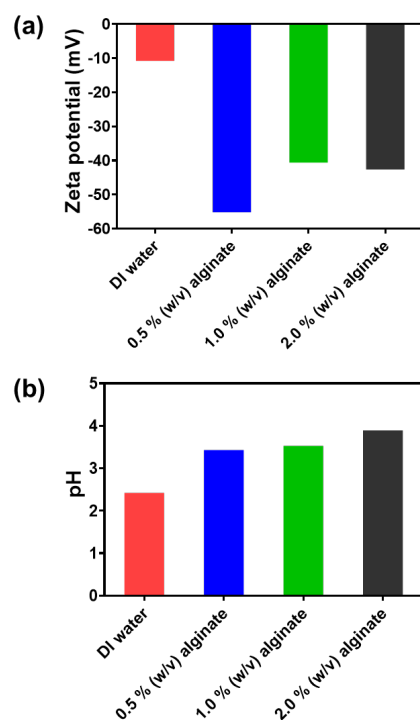


Figure S2.3. (a) Zeta potential and (b) pH of Au NPs suspensions prepared in DI water and alginate solutions with different concentrations (reaction time = 20 min)

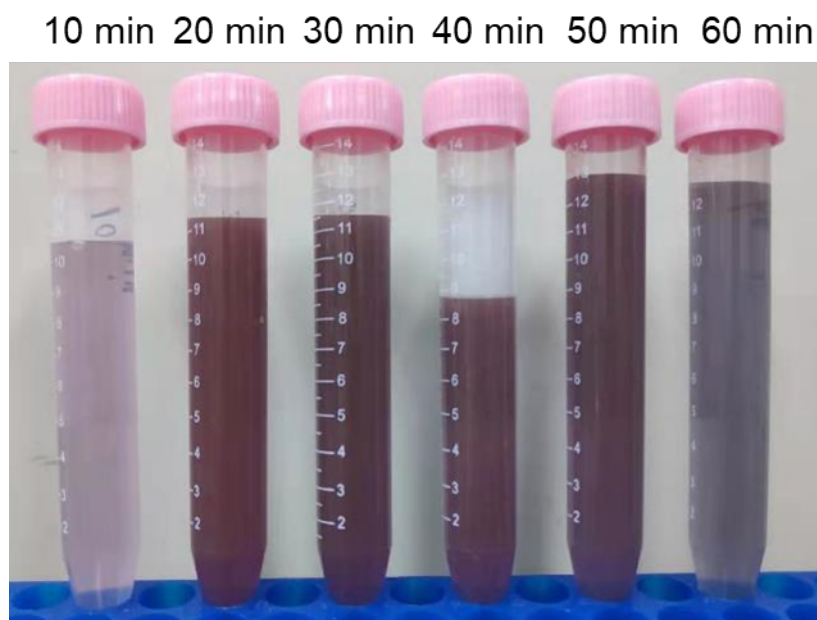


Figure S2.4. Au NPs prepared in DI water started to aggregate and deposit with increasing reaction times

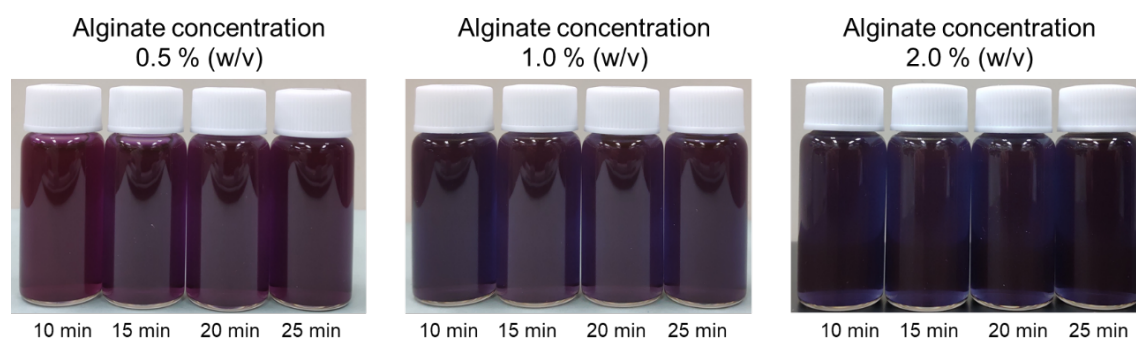


Figure S2.5. Au NPs prepared in different concentrations of alginate solutions with different reaction times

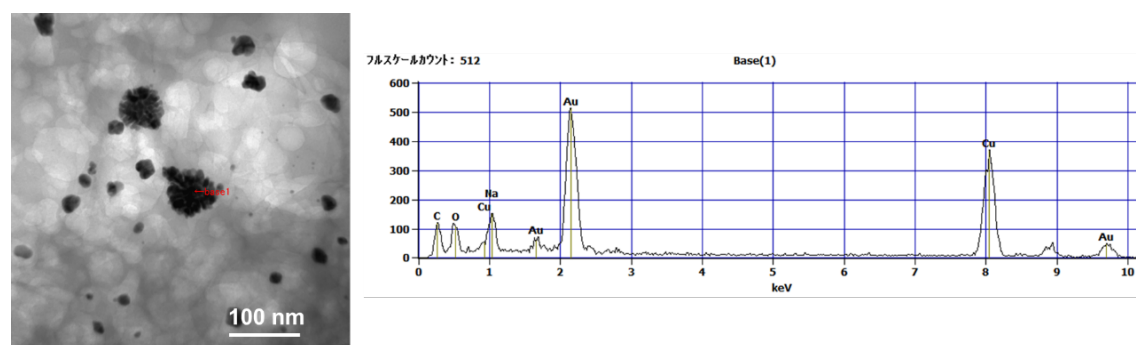


Figure S2.6. EDS results of Au aggregation (reaction time = 1 min) (alginate concentration: 2.0 % w/v)

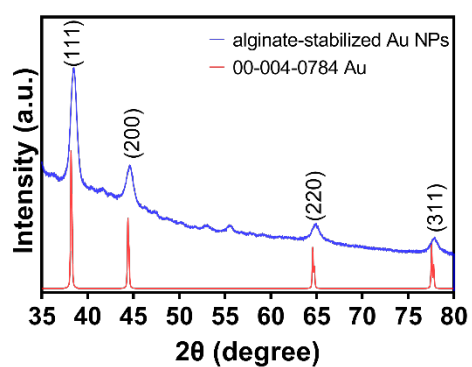


Figure S2.7. XRD result of alg-Au NPs (reaction time = 25 min) (alginate concentration: 2.0 % w/v)

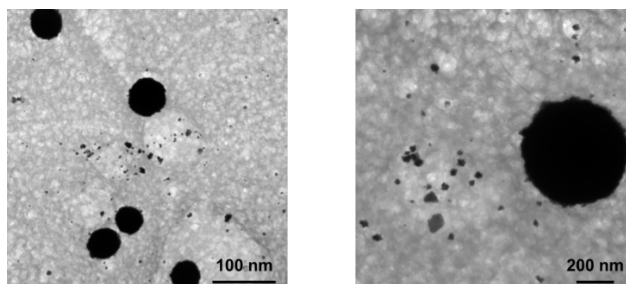


Figure S2.8. TEM images of Au aggregation in the in the early stage (reaction time = 1 min) of the reaction (alginate concentration: 1.0 % w/v).

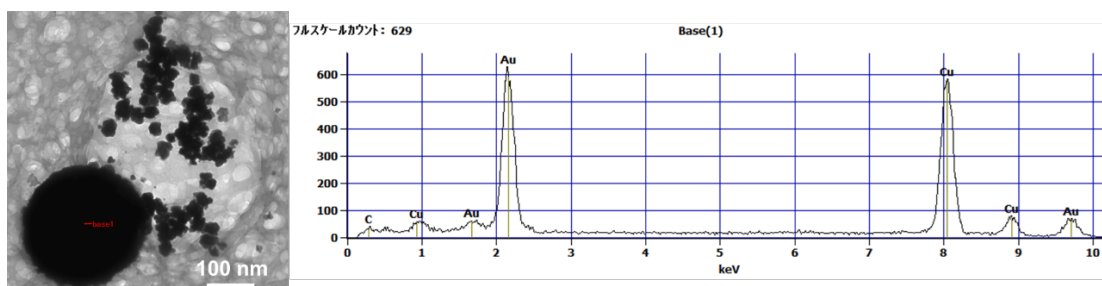


Figure S2.9. EDS results of Au aggregation (reaction time = 1 min) (alginate concentration: 1.0 % w/v)

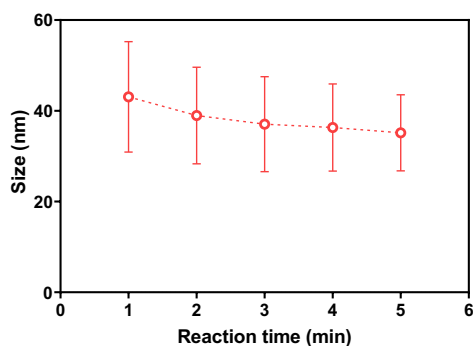


Figure S2.10. The size of Au NPs prepared in alginate solutions with different reaction time (alginate concentration of 1.0 % w/v)

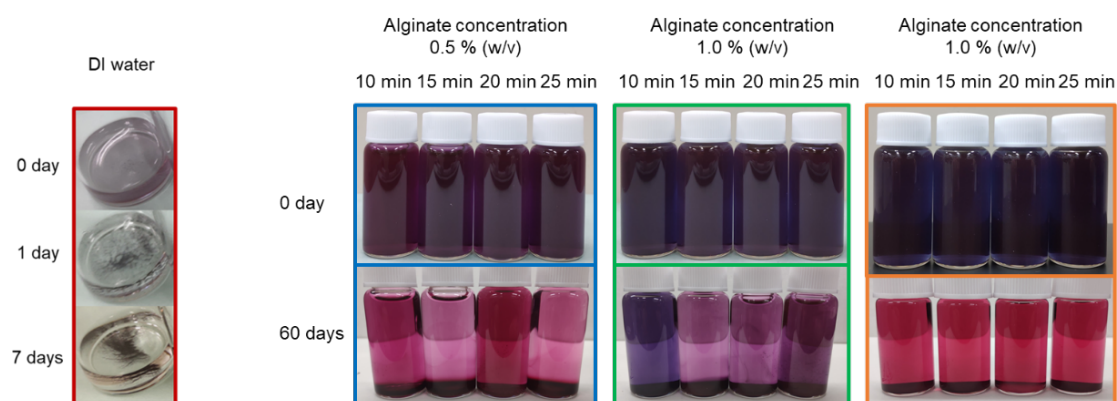


Figure S2.11. Images of Au NPs prepared in DI water on day 0, day 1 and day 7, and Au NPs prepared in alginate solutions with different concentrations on day 0 and day 60

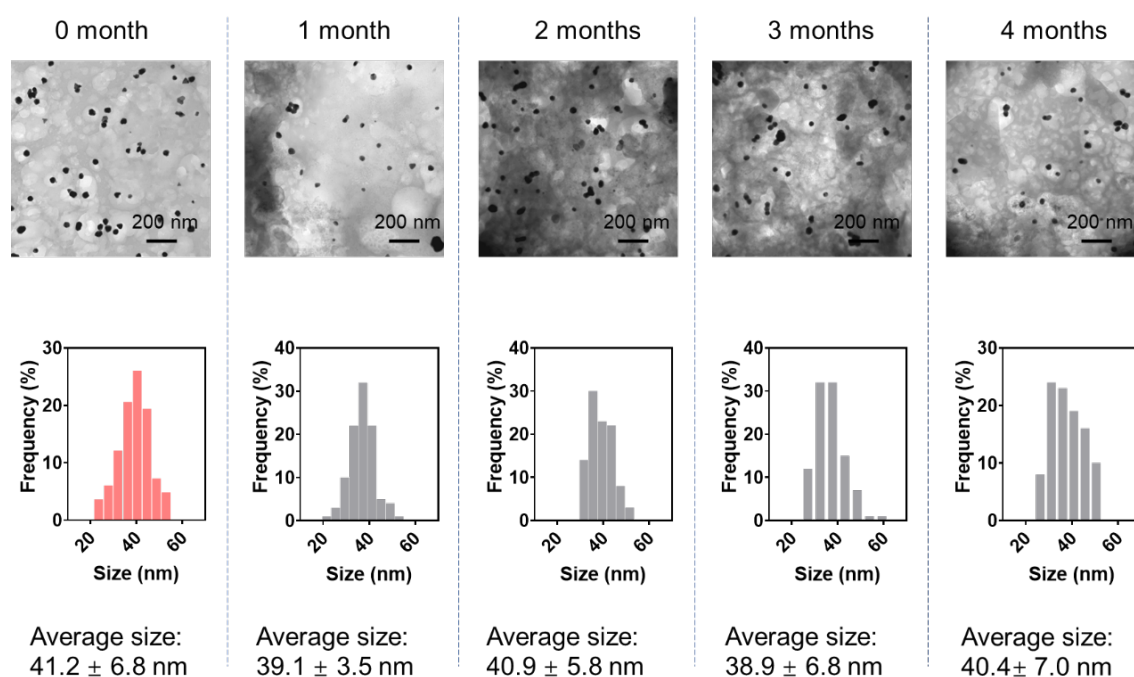


Figure S2.12. TEM and size distribution of I-1 alginate (0.5 % w/v)-stabilized Au NPs after accelerated stability test

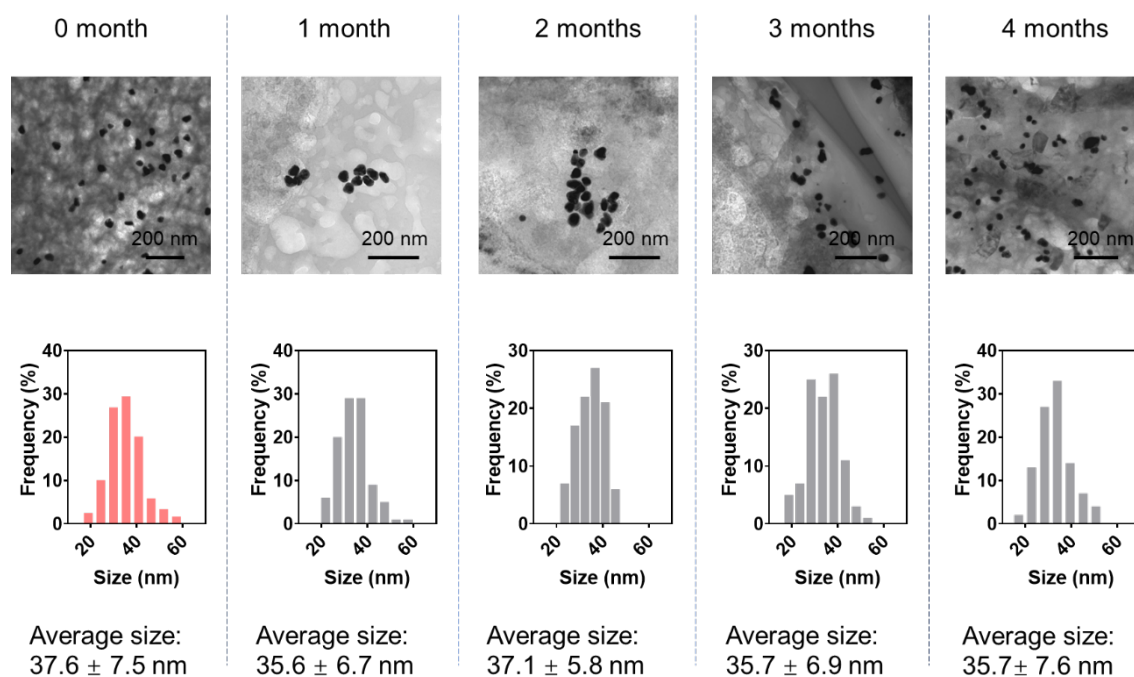


Figure S2.13. TEM and size distribution of I-1 alginate (1.0 % w/v)-stabilized Au NPs after accelerated stability test

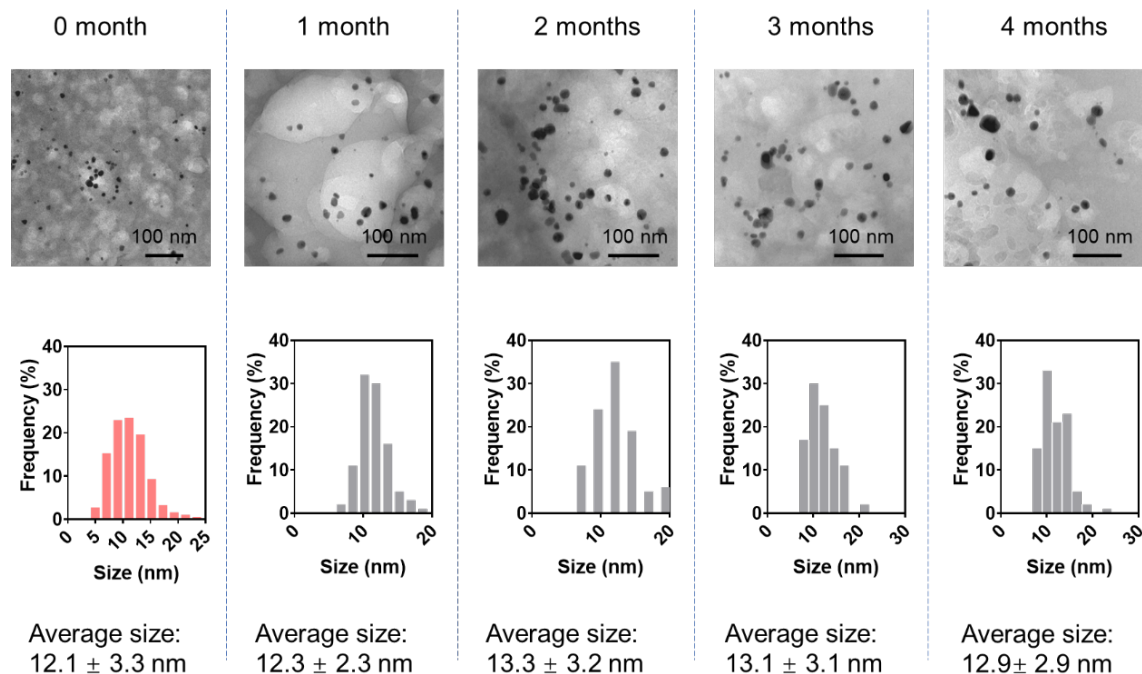


Figure S2.14. TEM and size distribution of I-1 alginate (2.0 % w/v)-stabilized Au NPs after accelerated stability test

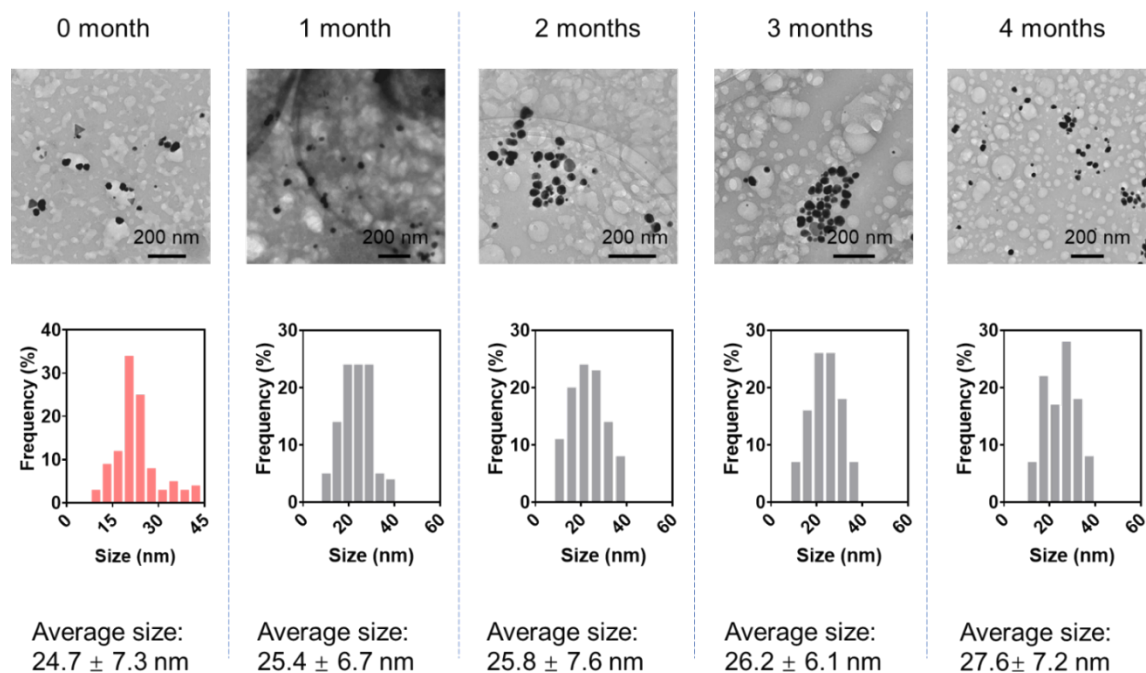


Figure S2.15. TEM and size distribution of I-8 alginate (0.5 % w/v)-stabilized Au NPs after accelerated stability test

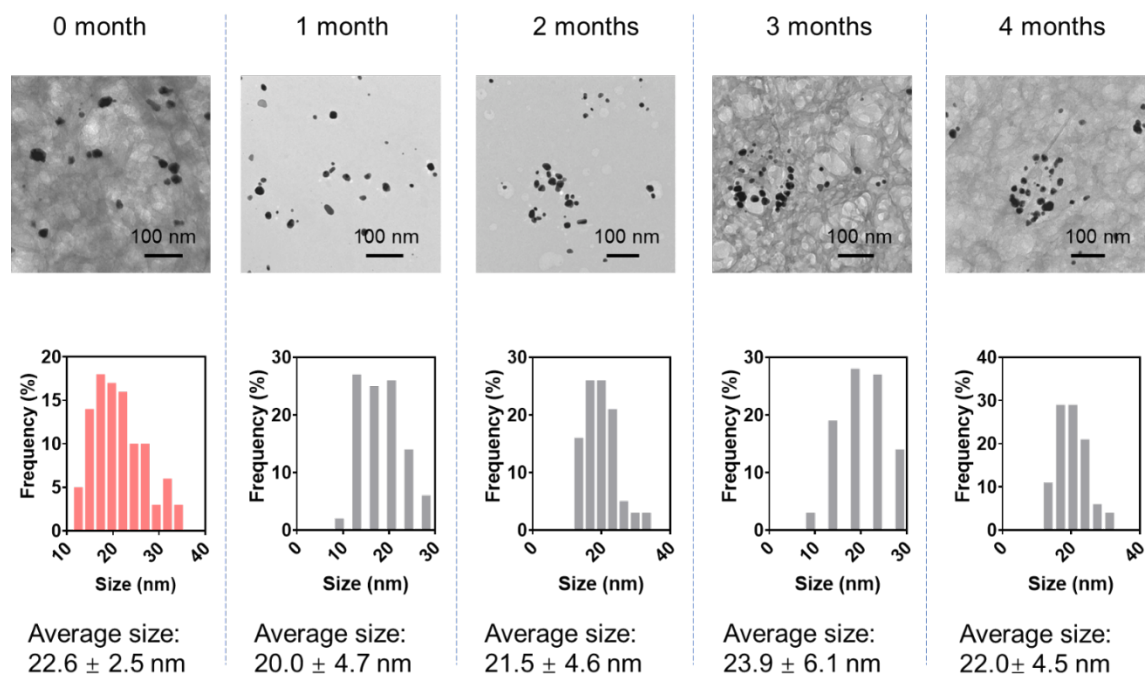


Figure S2.16. TEM and size distribution of I-8 alginate (1.0 % w/v)-stabilized Au NPs after accelerated stability test

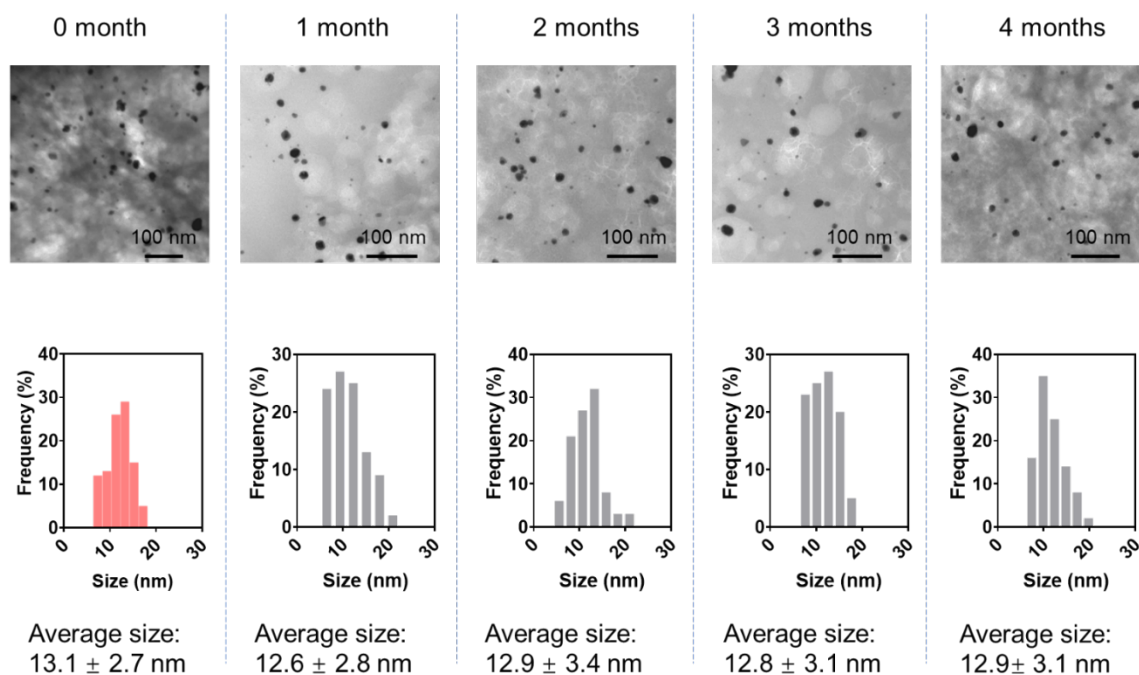


Figure S2.17. TEM and size distribution of I-8 alginate (2.0 % w/v)-stabilized Au NPs after accelerated stability test

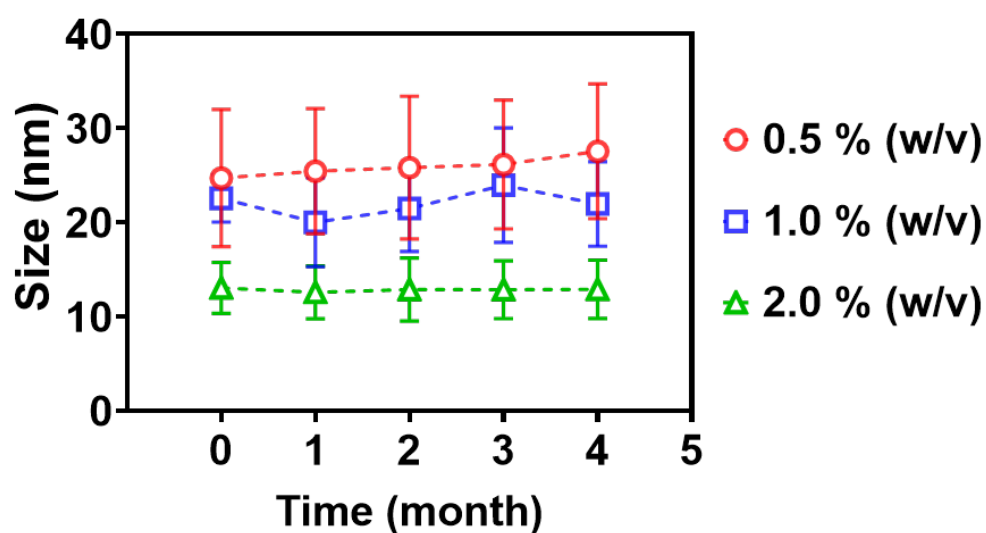


Figure. S18. Average size of I-8 alg-Au NPs after accelerated stability test

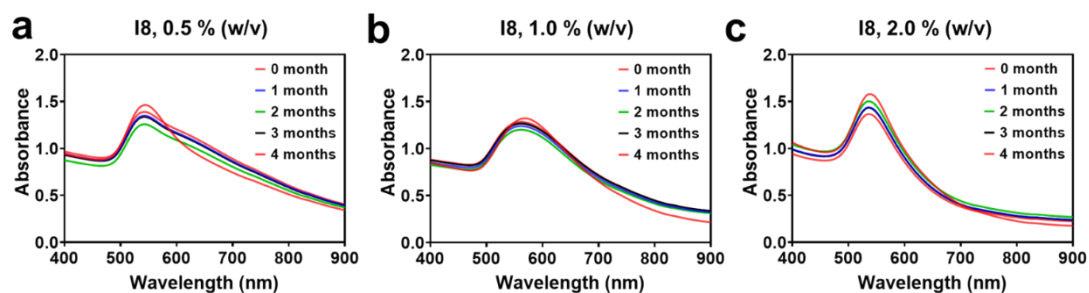


Figure S2.19. UV-Vis spectra of I-8 alg-Au NPs with different alginate concentrations ((a) 0.5% w/v, (b) 1.0% w/v, and (c) 2.0% w/v) after accelerated stability test

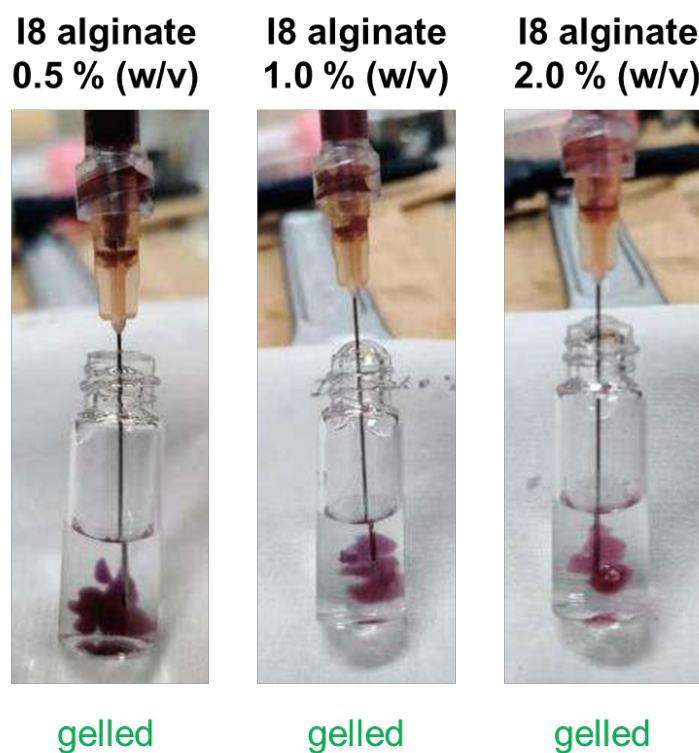


Figure S2.20. Injectability and gelation performance of I-8 alg-Au NPs in Ca^{2+} buffer (1.8 mM) after accelerated stability test

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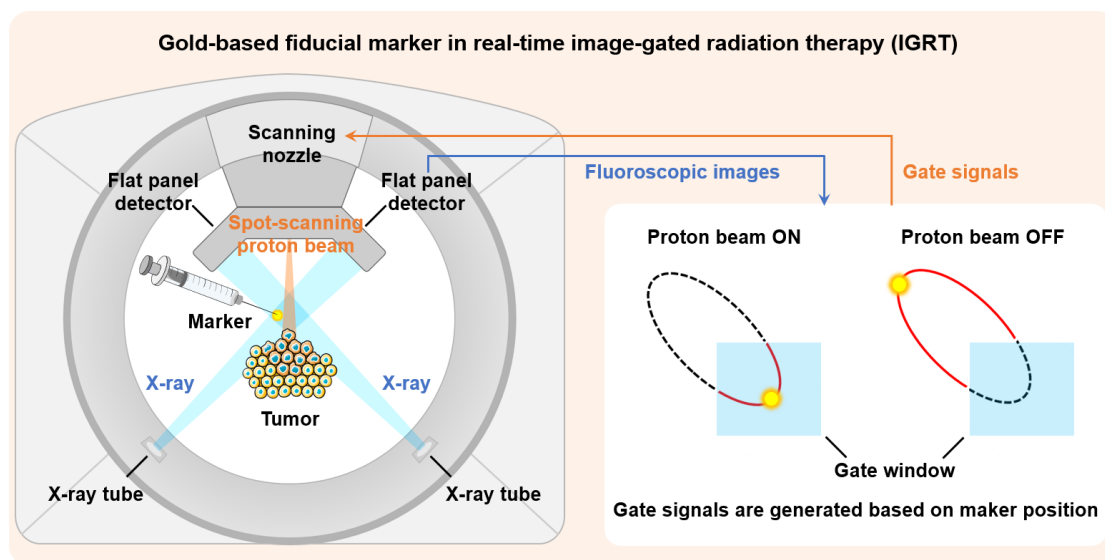
Chapter 3. Green And Effective Synthesis of Gold Nanoparticles as Injectable Fiducial Marker for Real-Time Image-Guided Radiation Therapy

Abstract

Fiducial markers are crucial for reducing damage to surrounding healthy tissues in real-time image-guided radiation therapy (IGRT) by boosting geometric accuracy and precision. This chapter aims to develop an injectable fiducial marker based on gold nanoparticles (Au NPs) synthesized by a green ethanol reduction method, as well as to determine the factors that affect their injectability and imaging capability. Au NPs synthesized in the ethanol-only system had an improved injectability and their imaging performance was almost identical to current fiducial markers used in the clinic. The reducing capacity of the ethanol system was significantly enhanced through the introduction of sodium alginate. Meanwhile, the addition of sodium alginate could also control the size of Au NPs, ranging from 21.8 to 14.1 nm. The product alg-Au NPs with smaller sizes enabled an injectability up to 100% even through 25G needles with an extrusion force less than 17 N, which is superior for the injection of high-concentration Au NPs. A better imaging performance of alg-Au NPs was also verified in the X-ray visualization test, exhibiting promise as a fiducial marker for IGRT.

3.1. Introduction

The excellent biocompatibility, low toxicity, easy surface functionalization, and interactions between substances[1] of gold with different types or structures make them potential candidates in diverse biomedical fields,[2, 3] including clinical diagnostics,[4, 5] therapies,[6, 7] biosensors,[8] immunology.[9, 10] In addition to these applications, the unique properties of gold, i.e., high atomic number, electron density, and surface plasmon resonance (SPR), also allow gold-based materials to be readily used for labelling and imaging, such as computed tomography (CT),[3, 11, 12] magnetic resonance imaging (MRI),[13] nuclear imaging,[14] optical imaging[15], photoacoustic imaging.[16] For example, gold-based fiducial markers are essential in real-time image-guided radiation therapy (IGRT).[17-19] By identifying the fiducial marker with X-ray beams, IGRT can accurately track the position of a tumor to avoid geometric uncertainties caused by breathing motion, peristalsis, and other body motions during therapy. As a result, IGRT is capable of improving the precision of radiation delivery, reducing the doses to the surrounding healthy tissues, and eventually achieving an optimal therapeutic efficacy and minimum treatment toxicity. However, currently used fiducial markers with large physical dimensions would lead to complicated and invasive insertion procedures, inducing undesired complications.[20-22] Obviously, injectable fiducial markers based on smaller-sized gold nanoparticles (Au NPs) are promising and preferred in IGRT to relieve patients' pain and improve their clinical experience (**Scheme 3.1**).[23-25]



Scheme 3.1. Illustration of gold-based fiducial marker in IGRT.

For the synthesis of Au NPs, fabrication methods can be categorized into two types: top-down and bottom-up approaches. Top-down approaches generally involve the synthesis of Au NPs starting from bulk materials to nanoscale architectures using various methods, such as laser ablation,[26, 27] sputtering,[28] irradiation,[29] aerosol technology,[30] or lithography.[31] However, top-down approaches are more susceptible to defects as the size becomes smaller and more expensive compared to bottom-up approaches.[32] Conversely, bottom-up approaches begin by forming structures at the atomic level. Of all the bottom-up approaches for synthesizing Au NPs, the chemical reduction methods are the most used strategies,[33] while the Turkevich method is the most classic and highly used chemical reduction technique for bio-materials,[34] in which gold ions in the precursor are reduced by reducing agents such as citrate,[35, 36] amino acid,[37] ascorbic acid,[38] or sodium borohydride.[39, 40] However, by-products may be generated by these agents during

reactions and cause unexpected biological toxicity.[41] For example, the presence of sodium citrate residues was proved to affect the proliferation and induce cytotoxicity in cells.[42, 43] Washing via centrifugation or water treatment are therefore indispensable.[44, 45] Furthermore, a highly controllable process protocol (temperature, reagent concentrations, and pH) must be strictly followed in order to synthesize monodisperse Au NPs with desired sizes.[46] Instead, low carbon-number alcohol reduction method was pioneered to synthesize noble metal NPs as catalysts for hydrogenations.[47-49] With the validated reduction capacity for mono- and bimetallic NPs synthesis,[50-52] as well as its environment-friendly and non-toxic properties, the alcohol reduction method represents a green alternative to manufacturing Au NPs

In this chapter, a facile, green, and biocompatible synthesis method based on the ethanol reduction system to prepare Au NPs for fiducial marker application was demonstrated. Initially, Au NPs were synthesized in the ethanol-only system and their injectability and imaging capabilities as fiducial markers were evaluated. The synthesized Au NPs had an improved injectability (through 18G and 21G needles) and an almost identical imaging capability compared to current fiducial markers used in the clinic. Due to the mild reducing capacity of ethanol and the lack of stabilizing agents, challenges such as low reaction rate, aggregation, and relatively large size were still faced in the synthesis of Au NPs and further injection through thinner needles (25G). Sodium alginate is a natural polymer with excellent biocompatibility, low toxicity. Owing to the abundant free hydroxyl and negatively charged

carboxyl groups, alginate has the potential to accelerate the reaction rate and control the growth of Au NPs. For this reason, the strategy of adding sodium alginate to the ethanol system was explored to tackle these limitations for higher efficacy and improved injectability. The reducing capacity of the ethanol system was significantly enhanced because of the introduced hydroxyl and carboxyl groups distributed along the alginate backbone.[53] Moreover, the size of Au NPs synthesized in the presence of sodium alginate (alg-Au NPs) could be regulated by the alginate chains-formed cavities and the increased nucleation probability. The average sizes of alg-Au NPs synthesized in different concentrations of alginate (0.1, 0.5, 0.7% w/v) were 21.8 ± 5.1 , 18.2 ± 3.0 , 14.1 ± 3.5 nm, respectively. Au NPs with smaller size enabled a superior injectability up to 100% even through 25G needles with extrusion force less than 17 N. As a result of increased gold surface area and more Au NPs distributed in the path of X-ray transmission, alg-Au NPs showed better imaging performance with the same gold concentration. Within the range of parameters studied, viscosities of injection media, Au NPs concentrations, needles gauges were found to be the major factors that would affect their injectability, while the gold amount and particle size dominated their imaging performance. This chapter describes a green and high efficiency reducing system and the potential of synthesized Au NPs as a fiducial marker for IGRT.

3.2. Experimental Section

3.2.1. Materials

Tetrachloroauric (III) acid hydrate ($\text{HAuCl}_4 \cdot n\text{H}_2\text{O}$, $n = 3.7$, Kojima, Japan), sodium alginate (alg, I-8, Kimica Corp., Japan), and anhydrous ethanol (Wako, compliant with Japanese Pharmacopoeia) were used as a precursor, an additive, and a reducing agent, respectively. 1 g of $\text{HAuCl}_4 \cdot n\text{H}_2\text{O}$ ($n = 3.7$) was dissolved in 100 mL of pure water to obtain a 24.6 mM aqueous solution of HAuCl_4 . All chemicals were used as received. The pure water (Organo Puric system, $>18.2 \text{ M}\Omega \cdot \text{cm}$) was used to prepare solutions. Injection of Au NPs was carried out using 1-mL plastic syringes with the diameter of 4.2 mm and the length of 104 mm.

3.2.2. Synthesis of Au NPs and alg-Au NPs

As shown in **Figure S3.1**, to synthesize Au NPs, 191.9 mL pure water and 200 mL ethanol were mixed into a 1 L two-necked flask and were heated in an oil bath. 8.1 mL aqueous solution of HAuCl_4 (24.6 mM) was added when the mixed solution boiled. The reaction solution was kept for 2 h under reflux and stirring at 800 rpm. When the reaction was completed, the products were collected after a 10-minute water-cooling and ultrasonic process. In order to synthesize alg-Au NPs, pure water was replaced with an equal volume of sodium alginate aqueous solution and the concentrations of original sodium alginate aqueous solutions were 0.1, 0.5, and 1.0% w/v. The obtained alg-Au NPs were labelled with the

original alginate concentrations: 0.1, 0.5, and 1.0% w/v. In Section 3.3.2.2, ethanol was replaced with an equal volume of pure water to obtain an alginate-only system to synthesize Au NPs. The Au NPs were still labelled with original alginate concentrations.

3.2.3. Characterizations

UV-Vis spectra were collected to observe Au NPs formation by using a UV-Vis spectrophotometer (UV-1800, Shimadzu) and a quartz cell with a 1-cm optical path. Transmission electron microscopy (TEM, JEOL JEM-2000-FX, at 200 kV) was used to analyse the morphology and size of alg-Au NPs. For TEM sample preparation, dispersions of Au NPs or alg-Au NPs were placed on collodion film-coated copper TEM grids and left for natural drying. Thermogravimetric (TG, DTA-60H, Shimadzu) analysis was carried out to confirm the mass ratio of gold to sodium alginate and to evaluate the thermal decomposition behaviour of samples. Fourier-transform infrared (FTIR) spectroscopy (Nicolet IS10, Thermo Fisher Scientific) was employed to analyse the functional groups of alginates before and after reactions in the range of 4000-500 cm^{-1} . The viscosities of sodium alginate aqueous solutions with different concentrations were measured by a viscometer (DV-I Prime, Brookfield) with shear rate sweeps from 4.0 to 200.0 s^{-1} at room temperature.

3.2.4. Injectability Tests

The injectability was evaluated by extraction forces and injectability ratios with an Automatic Vertical Servo Stand (JSV H1000, Algol Instrument). Briefly, as illustrated in

Figure S3.2, samples were loaded into a 1-mL syringe with an 18 G (inner diameter: 0.84 mm ϕ), 21G (inner diameter: 0.51 mm ϕ), or 25G (inner diameter: 0.26 mm ϕ) needle. The minimum forces to inject all samples (injectability: 100%) were recorded. The volume percentage of the extruded sample with respect to the total sample under a force of 50 N was defined as injectability. The threshold of the experimental platform was set to 70 N and the instrument would stop when the extraction force exceeded 70 N.

3.2.5. X-Ray Visualization Tests

In order to evaluate the X-ray visualization of samples, the X-ray visualization tests were conducted at the Central Institute of Isotope Science, Hokkaido University. **Figure S3.3** shows the schematic diagram of the X-ray visualization test. The samples were injected into jelly and 1 mL CaCl₂ solution (1.8 mM, physiological concentration) was reinjected for the gelation of each sample (alg-Au NPs). The gelated samples were taken out and irradiated with X-rays generated from an X-ray tube (UD-150-B40, Shimadzu). Gold ball markers (99.99% Au) with diameters of 1.5 and 2.0 mm were chosen as the control. The size and shape of the marker were determined to be suitable for pattern-recognition during marker tracking.[54] The X-rays passed through the samples would be detected by the Flat Panel Detector (PaxScan 3030, Varian Medical System). Besides, acrylic resins that have an X-ray attenuation property close to the human body were placed between samples and the detector,[55] and the visualization ability was evaluated by the pattern matching scores

quantified by the detector. The pattern matching scores were calculated according to a previous study.[56]

3.3. Results and Discussion

3.3.1. Au NPs Synthesized via Ethanol Reduction as Fiducial Marker

For various diagnostic and therapeutic applications, different types of reducing, capping, or stabilizing agents are required for the synthesis of Au NPs with desirable shape and size. Considering the *in vivo* usage scenarios of Au NPs and the potential toxicity of these agents, Au NPs were first synthesized via a one-step method using ethanol as a reducing agent.

3.3.1.1. Synthesis of Au NPs

Figure 3.1a shows the UV-Vis extinction spectrum of Au NPs synthesized by the ethanol reduction method in pure water/ethanol mixed solvent without any stabilizing molecules. Spectrum of Au NPs exhibited a surface plasmon resonance (SPR) peak with a maximum absorption wavelength (λ_{max}) at around 576 nm. Furthermore, another two SPR peaks centred at 738 and 833 nm were attributed to large hexagonal Au NPs, as evidenced by the TEM images in **Figure 3.1b** and **Figure S3.4**. When reductions occur on the surface of Au nucleated seeds, they depend on the surface energy of the different crystal facets. Therefore, polygonal particles would be generated during the reduction process.[57] **Figure 3.1c** shows the size distribution of Au NPs obtained from the arbitrarily chosen area of enlarged TEM images and their average size is approximately 242.5 ± 55.4 nm.

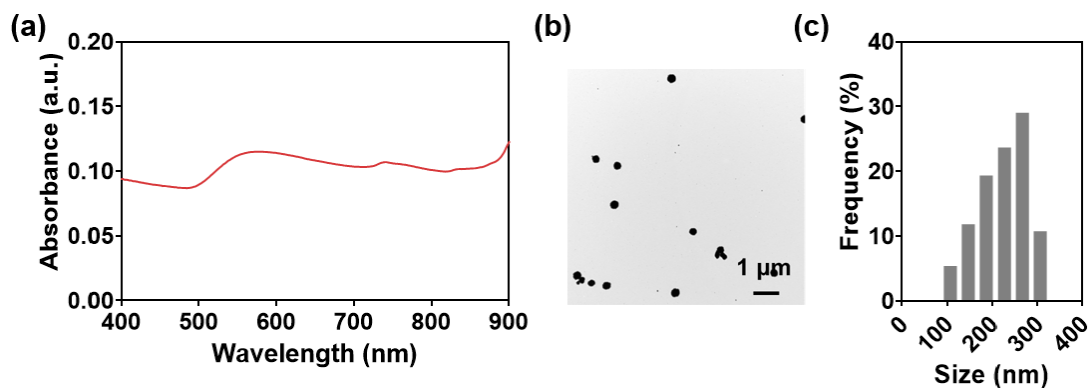


Figure 3.1. (a) UV-Vis spectrum, (b) TEM image, and (c) size distribution (taken from TEM image) of Au NPs synthesized via ethanol reduction method in pure water/ethanol mixed solvent without any stabilizing molecules

3.3.1.2. Injectability of Au NPs

High-efficiency transfer of Au NPs through injection guarantees superb image quality and the high injectability of fiducial markers is a key factor in improving patients' clinical experience by relieving pain during insertion procedures. Therefore, it is critical to study the injectability of Au NPs and to investigate the effect of relevant parameters (i.e., the viscosity of injection medium, Au NPs concentration, and needle gauge) on injectability.

Aqueous solution of sodium alginate was selected as injection medium in this study for its advantages such as ease of preparation, biocompatibility, biodegradability, and nontoxicity.[58] And the effect of injection medium viscosity (adjusted by altering alginate concentration, **Figure S3.5**) on injectability was firstly evaluated as the viscosity is vital for the efficient delivery of Au NPs and their uniform distribution in the injection medium.

Figure 3.2a shows the extrusion forces required for different concentrations of alginate aqueous solutions (1.0, 2.5, 5.0% w/v) as injection media. Extrusion force curves are normally characterized by a very rapid increase of the force followed by a steady stage. The average extrusion forces of steady stages are exhibited in **Figure 3.2b**. When the concentration of alginate aqueous solution was 1.0% w/v, the extrusion forces for 100% injectability were 0.7 N for an 18G needle, 0.8 N for a 21G needle, and 4.4 N for a 25G needle. When the alginate concentration increased to 2.5% w/v, the extrusion forces increased to 1.8, 3.0, and 9.2 N for the 18G, 21G, and 25G needles, respectively. A further 2-fold increase in alginate concentration (5% w/v) caused increases in extrusion forces to 5.3, 11.5, and 25.5 N for 18G, 21G, and 25G needles, respectively. The extrusion force required for injection is generally dissipated in three ways: (a) overcoming the resistance force between the syringe and plunger, (b) imparting kinetic energy to the injection medium, and (c) forcing the injection medium through the needle.[59, 60] The kinetic energy contributes to the flow of the injection medium while the driving force pushes the injection medium through the needle. Therefore, larger extrusion forces are needed for high-viscosity injection media and needles with smaller inner diameters.

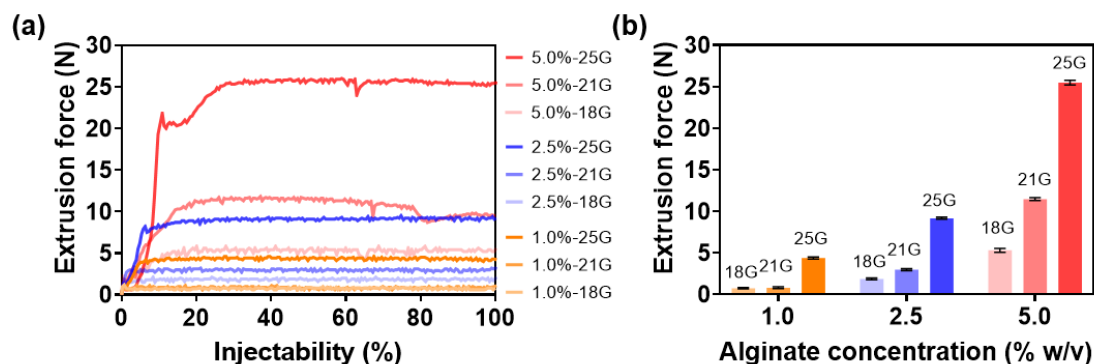


Figure 3.2. (a) Extrusion force curves and (b) average extrusion forces of injection media with different concentrations of aqueous solutions of sodium alginate when passing through different gauges needles.

After the introduction of Au NPs, the kinetic energy increased since the motion of contained Au NPs was driven by injection medium and required additional kinetic energy. On the other hand, due to potential congestion and clogging of Au NPs in the needle, the driving force of injection media with Au NPs also increased. Consequently, the introduction of Au NPs into the injection medium would increase the kinetic energy and driving force of the injection medium, which would further increase the extrusion force. Therefore, the Au NPs concentration is another important parameter that will strongly influence the injectability. In addition, the Au NPs concentration is also the key factor for imaging performance. Three different Au NPs concentrations (0.3, 0.5, and 0.7 g/mL) were consequently selected and evaluated for the balance of superior imaging performance and an extrusion force acceptable to the surgeon during injection. The extrusion forces were subsequently measured by altering the Au NPs concentrations in different viscosities of injection media through 18G needles. The injection system was a physical mixture of Au NPs and sodium alginate aqueous solution.

When using a low-viscosity sodium alginate aqueous solution (1.0 % w/v, viscosity = 170.0 mPa•s at a shear rate of 4 s^{-1} , **Figure S3.5**) as an injection medium, Au NPs particles would overcome the lateral drag force and deposit, resulting in no injectability. Instead, the deposition of Au NPs can be avoided since Au NPs cannot overcome the lateral drag force in high-viscosity sodium alginate aqueous solution (viscosities of 2.5 and 5.0 % w/v-sodium alginate solution are 9525 and 45244 mPa•s at a shear rate of 4 s^{-1} , **Figure S3.5**). The uniform distribution of Au NPs in the injection medium and the subsequent injectability can be therefore guaranteed. The alginate concentrations of injection media 2.5 and 5.0% w/v were consequently determined for the next injection tests. **Figure 3.3a** shows the extrusion forces of injection media with different concentrations of Au NPs. It was given by the red lines that the extrusion force surged when the Au NPs concentration was 0.7 g/mL. High concentration Au NPs increased the possibility of NP accumulation, and Au NPs were more likely to clog in the needles during injection, resulting in soaring extrusion forces and injectability less than 10%. In contrast, 100% injectability could be achieved at Au NPs concentrations of 0.3 and 0.5 g/mL (yellow and blue lines). Given that high concentration Au NPs can provide a better imaging performance, 0.5 g/mL was preferred for the following measurements. In particular, with the same concentration of Au NPs, the higher concentration of injection medium needed a higher extrusion force (**Figure 3.3b**), in agreement with the previous results (**Figure 3.2**). However, when the Au NPs concentration was 0.5 g/mL, the extrusion force for injection medium with 5.0% w/v of alginate concentration exceeded 50 N (grey dashed line), which

was not practical since 50 N is the maximum acceptable force for a surgeon to apply on a syringe during the injection.[61, 62] For this reason, although a high-concentration injection medium might facilitate the dispersion of Au NPs, the alginate concentration was ultimately determined to be 2.5 % w/v.

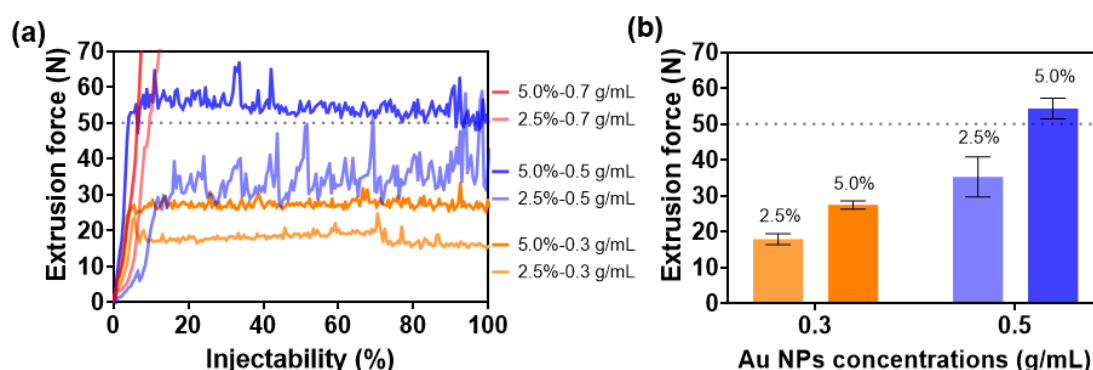


Figure 3.3. (a) Extrusion force curves and (b) average extrusion forces of different concentrations of Au NPs (synthesized by ethanol reduction in ethanol/water mixture without stabilizing molecules) of the injection medium with sodium alginate concentration. Needle gauge: 18G.

In addition to the viscosities of the injection media and Au NPs concentrations, the effect of needle gauges on injectability was also evaluated. Since the inner diameter of the 25G needle (0.26 mm ϕ) was too small for highly concentrated Au NPs with an average particle size of 242.5 ± 55.4 nm, no particles can flow through the needle, **Figure 3.4a** only shows the extrusion forces of the injection medium (alginate concentration: 2.5% w/v) with Au NPs (Au NPs concentration: 0.5 g/mL) through the 18G and 21G needles. The small diameter of needles made the flow of the injection medium with Au NPs through the needle more difficult,

as shown in **Figure 3.4b**, where one can see the average extrusion forces for 18G and 21G needles were 34.1 and 41.9 N, respectively. Furthermore, a thin needle also potentially increased the chance of needle blockage, inducing a dramatic increase in extrusion force and a decrease in injectability, by approximately 83.5 and 66.9% for 18G and 21G needles.

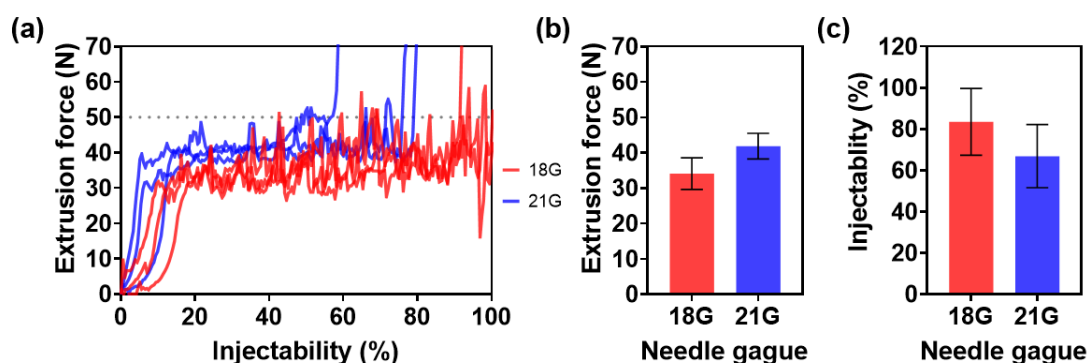


Figure 3.4. (a) Extrusion force curves, (b) average extrusion force, and (c) injectability (ratio of injected volume/total volume with extraction force of 50 N) through different needle gauges. Injection medium: sodium alginate with concentration of 2.5% w/v, Au NPs concentration: 0.5 g/mL.

3.3.1.3. Imaging Performance

Following the injectability evaluation, the imaging performance of samples contained different gold amounts (#1-7) and clinically used gold ball markers (1.5 and 2.0 mm ϕ) were tested by the X-ray visualization test. **Figure 3.5a** shows images obtained from an X-ray visualization test under different acrylic resin thicknesses and tube currents. The pattern matching scores calculated based on these images are displayed in **Table S3.1** and **Figure S3.6**. Pattern matching score is a quantitative measurement of the visibility of the object, and the maximum score is defined as 100. Samples with a score over 30 are considered

sufficiently visible. To intuitively compare the imaging performance of Au NPs and gold ball marker, the pattern matching scores of Au NPs were normalized by dividing them with the pattern matching score of the 2.0-mm gold ball marker under the same acrylic resin thickness and tube current, and the gold amounts contained in samples were calculated based on their TG analysis, as exhibited in **Figure 3.5b** and **Figure S3.7**. Under a thin acrylic resin (10 cm), samples containing a small amount of Au NPs (15.00 mg) showed acceptable imaging capability. However, the pattern matching scores decreased with increasing acrylic resin thickness, suggesting that the human tissue would also cause the attenuation of X-ray and affect the *in vivo* visibility of Au NPs as a fiducial marker. Higher pattern matching scores under thicker acrylic resin could be achieved by raising the tube current to improve their imaging performance deep inside the human body. For samples containing more gold, the higher gold amount also endowed them with enhanced imaging capability under thicker acrylic resins. In particular, sample #5 could reach a similar imaging level (all normalized pattern matching score larger than 80%) with a smaller gold amount (29.87 mg) compared to the 2.00-mm gold ball marker (80.95 mg). These results demonstrate that the Au NPs synthesized via the ethanol reduction is capable of being a fiducial marker for an imaging application.

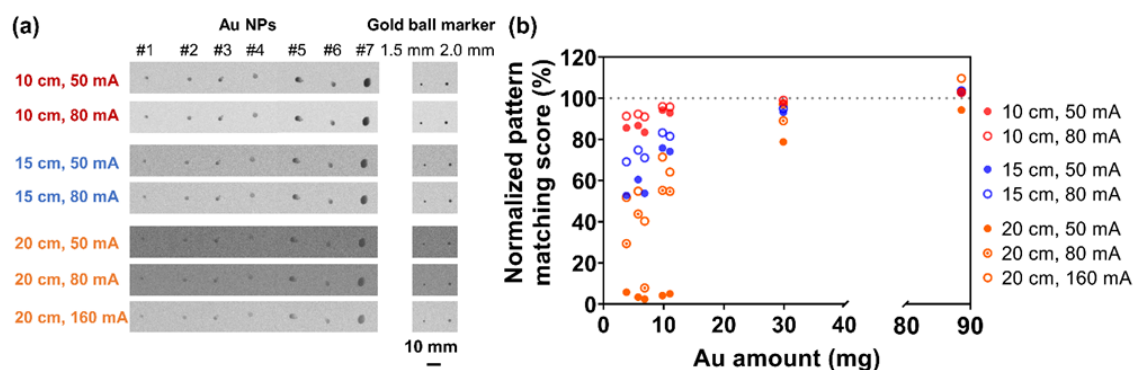


Figure 3.5. (a) X-ray visualization image of gold ball markers and samples with different amounts of Au NPs under different acrylic resin thicknesses and tube currents. (b) Relationship between normalized pattern matching scores and different gold amounts.

3.3.2. Alg-Au NPs Synthesized via Ethanol Reduction with Alginate as Fiducial Marker

Ethanol reduction is a green method for the synthesis of Au NPs without the addition of toxic reducing and stabilizing agents. However, the challenges of low reaction rate, excessive reaction-induced aggregation and deposition, and the relatively large particle size still remain because of the mild reducing capacity of ethanol and the long reaction time. Especially, the aggregation between Au NPs and their large size would further increase the risk of needle clogging during injections, leading to zero injectability through thin needles (25G) and inefficient transfer through larger gauge needles (**Figure 3.4**). Owing to the abundant free hydroxyl and carboxyl groups distributed along the backbone and interpenetrating chains after being dissolved in solution,[63] sodium alginate might have the potential to accelerate the reaction rate and control the growth of Au NPs. For this reason, sodium alginate was

introduced into the green reduction system for a high-efficiency synthesis of Au NPs with controlled particle sizes.

3.3.2.1. Synthesis of Alg-Au NPs

As shown in **Figure 3.6a**, the formations of alg-Au NPs in different concentrations of alginate were confirmed by the UV-vis spectra, in which SPR peaks with λ_{max} around 535 nm could be observed. In comparison with the UV-vis spectrum of Au NPs synthesized in the ethanol-only system, the spectra of alg-Au NPs displayed an ideal bell shape, indicating that negligible aggregation occurred, and the Au NPs were well dispersed. Moreover, according to the spectra, there were only single SPR peaks around 535 nm, which indicates that very few hexagonal or triangular Au NPs were generated in the ethanol/alginate system. This could also be verified by their TEM images.

Figure 3.6b shows the TEM images and their size distributions of Au NPs synthesized in the ethanol/alginate system with various alginate concentrations and most alg-Au NPs were spherical. Meanwhile, in **Figure 3.6c**, the decreasing particle size of alg-Au NPs with the increasing alginate concentration can be observed based on their particle size distributions. The cavities formed by alginate chains could serve as a template to limit the uncontrollable growth of Au NPs. As a result, Au NPs synthesized in a higher concentration of alginate aqueous solutions would have a smaller particle size. The size of Au NPs is critically important to their properties and biomedical applications, including biodistribution,[64]

metabolism,[65] permeation,[66] cytotoxicity,[67] and cell uptake.[68] Radiosensitization,[69] X-ray attenuation,[70] and targeted delivery[71] are also size-dependent. Therefore, in addition to reducing the risk of needle clogging, the use of Au NPs can also be extended to other biomedical applications by controlling the size of Au NPs via regulating the alginate concentration.

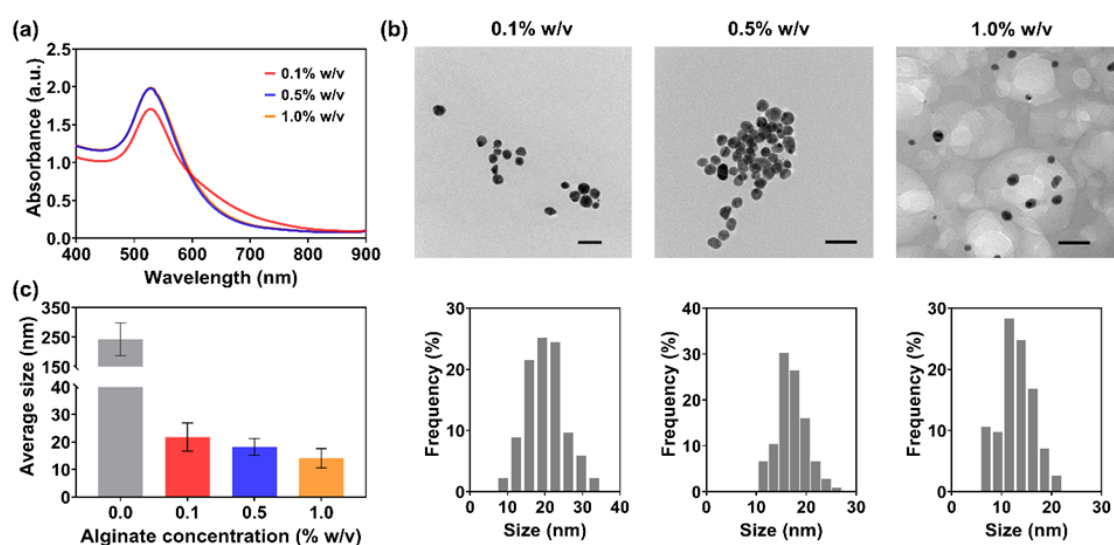


Figure 3.6. (a) UV-Vis spectra, (b) TEM images and size distributions, and (c) average sizes of alg-Au NPs synthesized with different alginate concentrations. The scale bars shown in all TEM images indicate 50 nm.

3.3.2.2. Formation of Alg-Au NPs in the Ethanol/Alginate System

As expected, the addition of alginate was capable of facilitating the reduction reaction, as validated by the start time of the reaction (the time when solution the color changed) in

Figure 3.7. The reaction almost started immediately when the alginate concentration was 1.0% w/v, exhibiting the excellent reducing capacity of the ethanol/alginate system. Even though

the alginate concentration decreased to 0.1% w/v, the start time of the reaction was still shorter than that of the ethanol-only system, which normally required at least 1 hour for the formation of Au NPs. In order to investigate the formation process of Au NPs in the ethanol/alginate system, Au NPs synthesized with 0.5% w/v alginate aqueous solutions were collected at different time points for further characterizations.

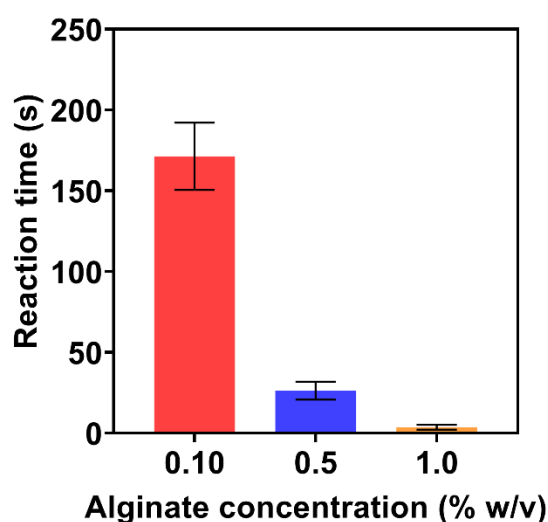


Figure 3.7. Start time of the reactions in the ethanol/alginate systems with different concentrations of alginate.

In **Figure 3.8a**, the SPR peak of Au could be observed at 30 s, which confirmed the formation of Au NPs. Thereafter, the absorption intensity increased with reaction time and had reached a relatively stable level since 60 s. Remarkably, the absorption intensity at 150 s was almost the same as the intensity after 2 h. These UV-vis spectra clearly demonstrate that Au NPs had formed within 30 s in the ethanol/alginate system and the reaction from Au(III) to Au(0) was basically completed after 150 s. **Figure 3.8b** and **Figure S3.8** represent the

TEM images and their size distributions of Au NPs at different time points, and their average sizes are shown in **Figure 3.8c**. The average sizes of the synthesized Au NPs did not change significantly at different time points and were all around 18 nm, suggesting that the chain-like structure and formed cavities can indeed limit the growth of Au NPs and prevent them from aggregation and deposition induced by the long reaction time. Furthermore, the coefficient of variation (CV) of Au NPs (**Figure 3.8d**), which is defined as the ratio of their standard deviation to the average size, gradually decreased with reaction time, which implied that the size of Au NPs became more uniform as the reaction progressed.

The FTIR spectra of sodium alginate and alg-Au NPs were measured to confirm if sodium alginate was involved in the reaction. As shown in **Figure 3.8e**, all FTIR spectra show broad peaks at ca. 3500 cm^{-1} , which are attributed to the stretching of hydroxyl groups due to the presence of water. Low-intensity peaks at about 2900 cm^{-1} are due to the $-\text{CH}_2$ groups. The absorption bands corresponding to the asymmetric and symmetric stretching modes of the carboxylate salt groups ($-\text{COONa}$) are located at 1610 cm^{-1} and 1417 cm^{-1} . The absorption bands at 1304 cm^{-1} are ascribed to the asymmetrical stretching of the C-O-C group while the vibrations in the range $1100\text{-}1000\text{ cm}^{-1}$ correspond to the glycoside bonds in the polysaccharide (stretching C-O-C). Peaks at 946 cm^{-1} , 890 cm^{-1} , and 818 cm^{-1} are attributable to the C-H vibration of the pyranose (ring of the alginate) group.[72] By comparing the FTIR spectra of sodium alginate and alg-Au NPs, the appearance of a new band at 1730 cm^{-1}

indicates the presence of a six-membered cyclic ketone, revealing a process by which the -OH groups underwent oxidation to C=O during the reaction.

Generally, sodium alginate itself can function as a reducing agent for nanoparticle synthesis.[73-75] To confirm the role of sodium alginate in the ethanol/alginate system, additional experiments were conducted by synthesizing Au NPs in the alginate-only solution under the same conditions. As shown in **Figure S3.9**, the alginate-only system also lacked efficiency. For the Au NPs synthesized in the 1% w/v alginate aqueous solutions, the reaction required a long reaction time (more than 2 h). When the concentration decreased to 0.5% w/v, the weakened absorbance compared to the 1% w/v group implied only partial Au(III) were reduced. Au NPs could not even form in a 0.1% w/v alginate aqueous solution. Given the weaker reducing capacity of sodium alginate and its lower concentration, ethanol is considered to play a leading role in the ethanol/alginate system where Au(III) were reduced with the oxidation of ethanol to aldehyde and carboxylic acid (**Scheme 3.2a**) [47, 48, 76]. Ethanol initially coordinates to Au(III) to generate an oxonium-complex. The resulting oxonium-complex would lose a proton and yield ethoxide-complex. Subsequent β -hydride elimination generates the hydride-complex, which then undergoes further deprotonation to yield a low valent Au ion and is eventually reduced to zero-valent state through disproportionation. In general, the reduction of Au(0) from Au(III) accompanies with the oxidation of ethanol into acetaldehyde, and the formation of acetaldehyde during the process is supported by the works from Hirai et al., in which the formation of acetaldehyde were

detected by gas-liquid partition chromatography (GLPC)[48] and chromotropic acid-sulfuric acid method,[49] respectively. The reaction equation is shown below:



As a supplementary role, sodium alginate provided carboxy anions and extra reducing groups to the ethanol system. As illustrated in **Scheme 3.2b**, Au(III) could be evenly dispersed in ethanol/alginate solution by chelating gold chloride anionic complexes with oxygen atoms from carboxyl and hydroxyl groups of alginate.[77] The addition of sodium alginate increased the number of groups involved in the reaction (**Figure 3.8e**), which enhanced the reducing capacity of ethanol system and promoted the reduction rate of Au(III) to Au(0). Meanwhile, due to the high reduction rate, a larger number of nuclei would be generated, which caused the formation of Au NPs with smaller sizes.[78] Therefore, the high nucleation probability in this highly reductive system is another factor that can control the size of synthesized Au NPs in addition to the alginate chains-formed cavities. In contrast, in the ethanol-only system, due to the longer reaction time, small particles shrank and disappeared, while large particles absorbed the atoms from the smaller dissolving particles and continued growing, which followed the Ostwald ripening model.[79] As a result of the lack of stabilizing and capping agents, the assembly and merging between Au NPs were inevitable, and large-sized Au NPs formed consequently.[80, 81]

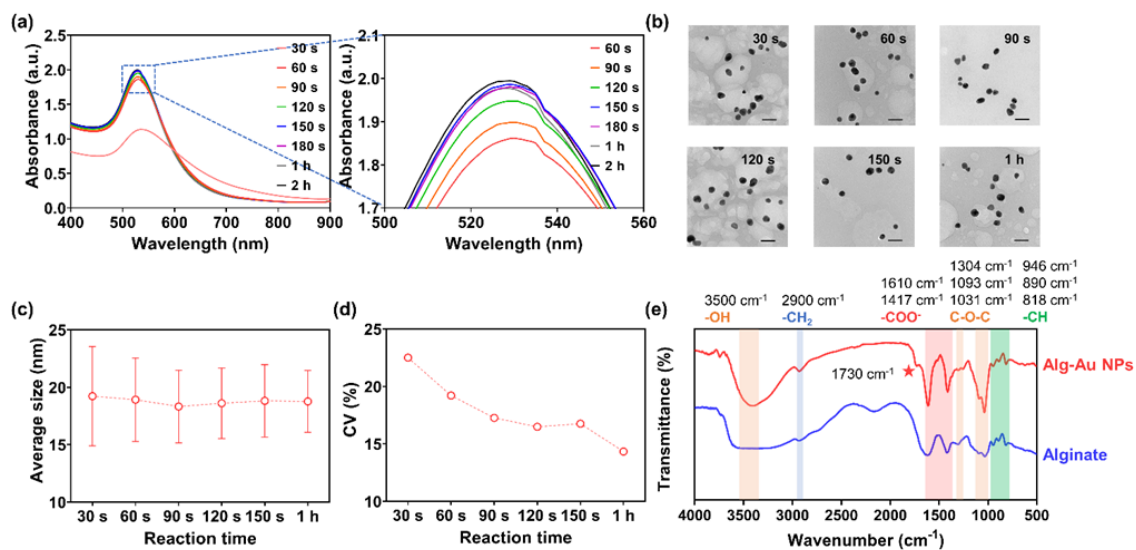
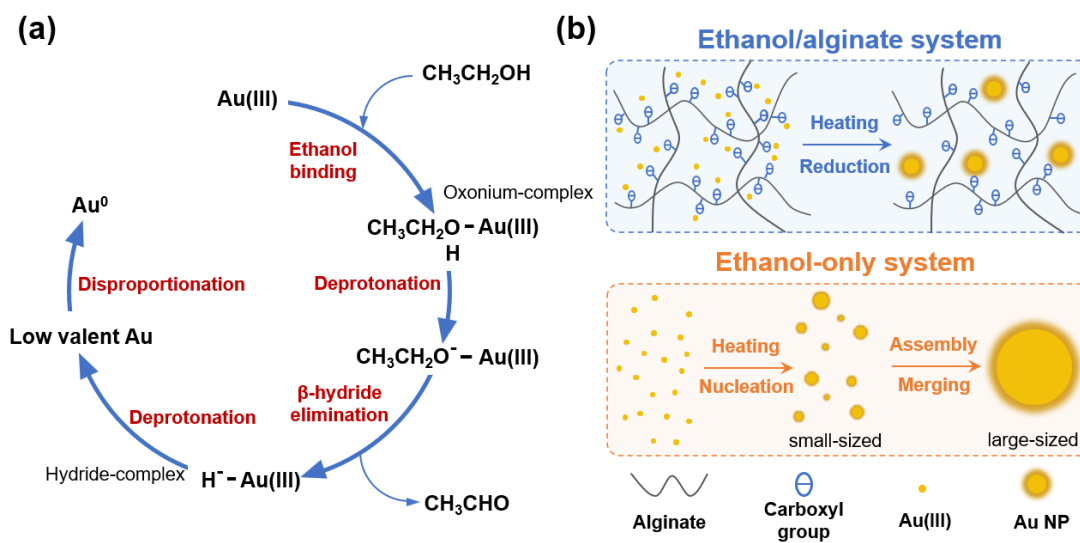


Figure 3.8. (a) UV-Vis spectrum, (b) TEM, (c) average sizes, and (d) CV of alg-Au NPs synthesized with 0.5% w/v alginate aqueous solutions. (e) FTIR of alg-Au NPs and alginate. The scale bar is 50 nm.



Scheme 3.2. Schematic illustration of (a) Ethanol reduction method and (b) formation process of Au NPs in the ethanol/alginate and ethanol-only system.

3.3.2.3. Injectability of Alg-Au NPs

In the synthesis process, the ratios of Au NPs to sodium alginate were fixed. Since the Au NPs amount is critical to the imaging capability, a higher amount of Au NPs could provide a better imaging quality. Paradoxically, a higher Au NPs amount would be accompanied by more sodium alginate, which would lead to an increased extraction force during the injection (**Figure 3.2**). Therefore, removal of sodium alginate while maintaining the properties of Au NPs (e.g., size and dispersibility) is necessary before injectability measurement. As sodium alginate can be decomposed by a high temperature,[82] sodium alginate was removed by thermal treatment in this study. Based on the sodium alginate decomposition behavior at different temperatures, the thermal treatment temperature was set at 400 °C because higher temperatures (600 °C) would cause the sintering between Au NPs (**Figure S3.10**). In **Figure S3.11**, from the TEM images, it is evident that the morphology of the Au NPs did not change significantly after thermal treatment. A few large-sized Au NPs could also be observed due to the sintering caused by the high temperature, which led to a slight increase in the average size. However, the amount of sintered Au NPs and the increased average size are negligible and acceptable. By comparing the TG curves before and after the thermal treatment, there was essentially no weight loss after the treatment, confirming the effective removal of sodium alginate via thermal treatment.

After the synthesis of alg-Au NPs and the removal of excess sodium alginate, the injectability of alg-Au NPs was studied with the optimal injection conditions in Section

3.3.1.2, in which the injection medium concentration was 2.5% w/v and the Au NPs concentration was 0.5 g/mL. As shown in **Figure 3.9**, the average extrusion forces required for Au NPs synthesized with different concentrations of alginate to pass through different gauge needles were: 3.5, 3.6, and 3.2 N for 18G needles (inner diameter: 0.84 mm); 6.9, 6.6, and 6.4 N for 21G needles (inner diameter: 0.51 mm); and 14.9, 14.5, and 14.1 N for 25G needles (inner diameter: 0.26 mm). As mentioned in Section 3.3.1.2, a portion of the extrusion force is responsible for forcing the injection medium through the needle, accounting for needles with smaller inner diameters requiring larger extrusion forces. However, all extrusion forces are less than 50N, which is extremely user-friendly for the surgeon during surgery. It is also worth noting that the alg-Au NPs synthesized in 0.1, 0.5, and 1.0% w/v alginate aqueous solutions with average sizes of 21.8 ± 5.1 , 18.2 ± 3.0 , and 14.1 ± 3.5 nm can all pass through different gauge needles, even the injectability through the 25G needle were also 100%. These results suggest that the injectability of Au NPs had been significantly improved by controlling the size of Au NPs with the addition of sodium alginate.

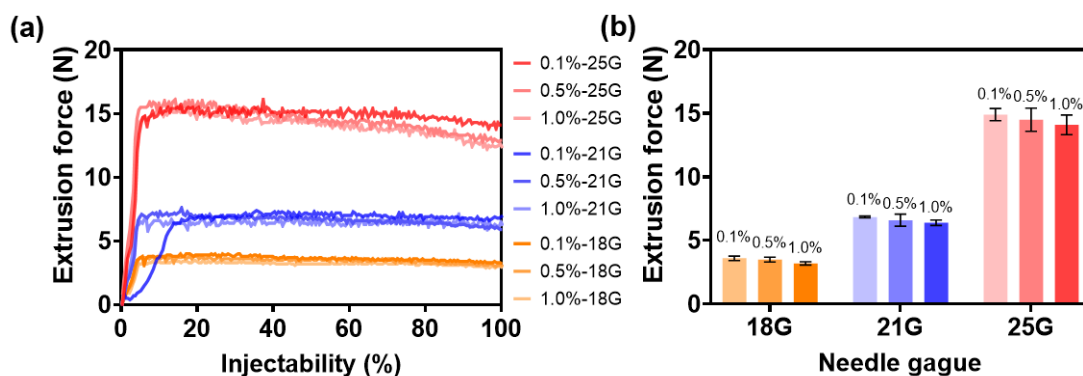


Figure 3.9. (a) Extrusion force curves and (b) average extrusion force of alg-Au NPs synthesized in different alginate concentrations through different needles.

3.3.2.4. Imaging Performance

Figure 3.10a and **Table S3.2** shows X-ray visualization test images of alg-Au NPs and the pattern matching scores are displayed in **Table. S3.2** and **Figure S3.12**. The amounts of gold contained in samples were calculated based on their TG analysis, as shown in **Figure S3.13**. Since the size difference between alg-Au NPs was not significant (14.1 to 21.8 nm) compared to Au NPs synthesized in the ethanol-only system (242.5 nm), the normalized pattern matching scores (**Figure 3.10b**) reveals that the gold amount, rather than the average size, was the major factor in determining the imaging capability. However, the alg-Au NPs with smaller sizes showed higher normalized pattern matching scores (over 100%) with lower gold amount (about 20 mg). This can be attributed to the increased surface area with the decrease in size. With the same gold concentration, smaller Au NPs could offer a larger gold surface area. Since the X-ray attenuation is target area dependent, small Au NPs with a larger surface area would exhibit more dramatic X-ray attenuation.[70, 83] Meanwhile, the smaller

size implied that a large number of Au NPs would distribute in the path of X-ray transmission, which increased the absorption or deflection (scatter) of photons and eventually led to X-ray attenuation as well.[84]

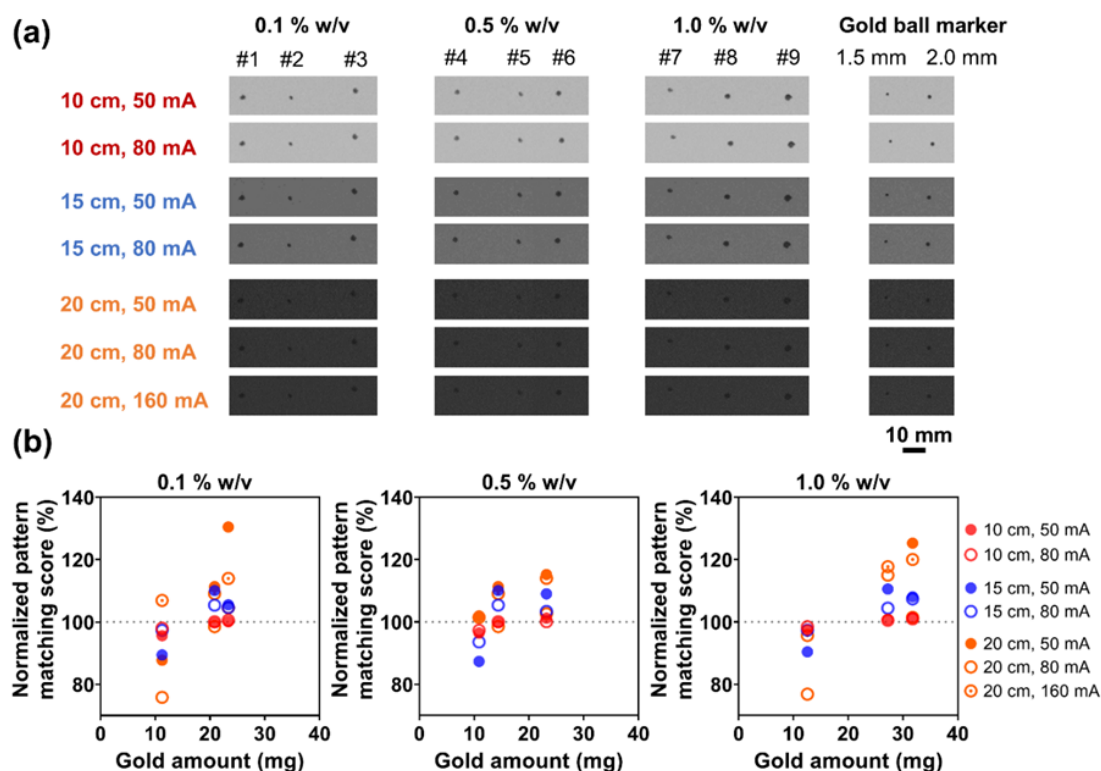


Figure 3.10. (a) X-ray visualization image of alg-Au NPs and gold ball markers under different acrylic resin thicknesses and tube currents. (b) Relationship between normalized pattern matching scores and different gold amounts.

3.4. Conclusions

In summary, Au NPs designed as injectable fiducial markers for IGRT were synthesized via ethanol-only and ethanol/alginate systems, respectively. Their injectability and imaging capabilities as fiducial markers were evaluated. Within the range of parameters studied, injection medium viscosity, Au NPs concentration, needles gauge was found to be the major

factors that would affect their injectability, while the gold amount and particle size dominated their imaging performance. Compared to conventional gold ball markers (1.5 or 2.0 mm ϕ), Au NPs synthesized in the ethanol-only system had an improved injectability (through 18G and 21G needles) and an almost identical imaging capability. This chapter also reports a novel reducing system based on the mixture of ethanol and alginate. The addition of alginate into the ethanol reduction system can improve the reaction efficiency, control the size of synthesized Au NPs, and prevent aggregation between Au NPs. As an anionic polymer with high charge density, alginate can significantly enhance the reducing capacity of the ethanol system and control the size of alg-Au NPs through the formed cavities and the increased nucleation probability. Due to the size control during synthesis, the alg-Au NPs with smaller sizes (14.1 to 21.8 nm) exhibited a superior injectability, up to 100% even through 25G needles with extrusion force less than 17 N, especially for the injection of high-concentration Au NPs. A better imaging capability of alg-Au NPs was also verified in the X-ray visualization test. Though the imaging performance of Au NPs and alg-Au NPs as fiducial markers has been examined and confirmed after injection.

3.5. Supporting Information

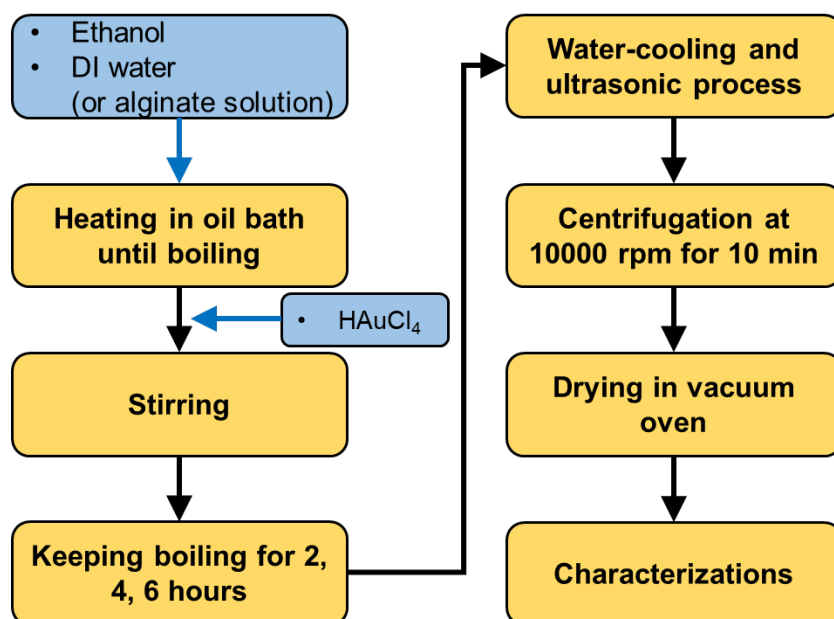
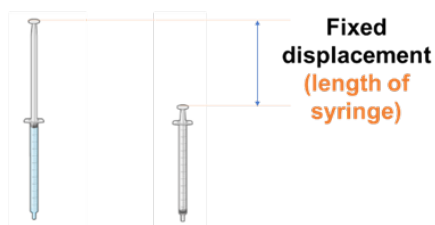


Figure S3.1. Synthesis route of Au NPs and alg-Au NPs.

1. Fixed displacement

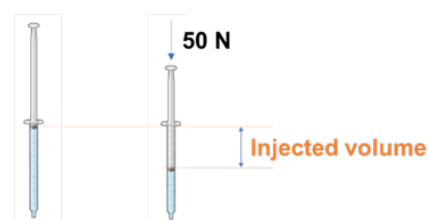
Minimum force required for injecting all samples



➡ Minimum force to inject all samples

2. Fixed force

How much samples could be injected



➡ Injectability = $\frac{\text{Injected volume}}{\text{Total volume}} \times 100 \%$

Figure S3.2. Illustration of injectability test.

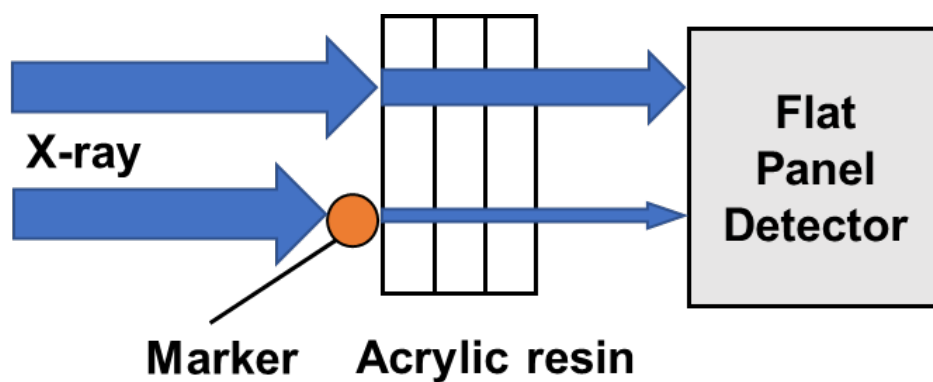


Figure S3.3. Schematic diagram of the X-ray visualization test.

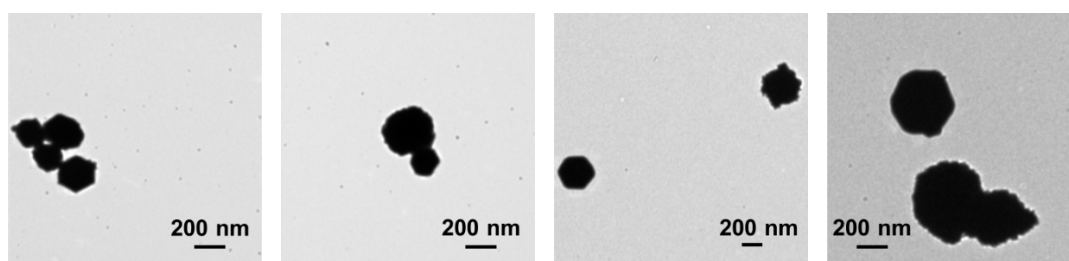


Figure S3.4. TEM of Au NPs synthesized via ethanol reduction.

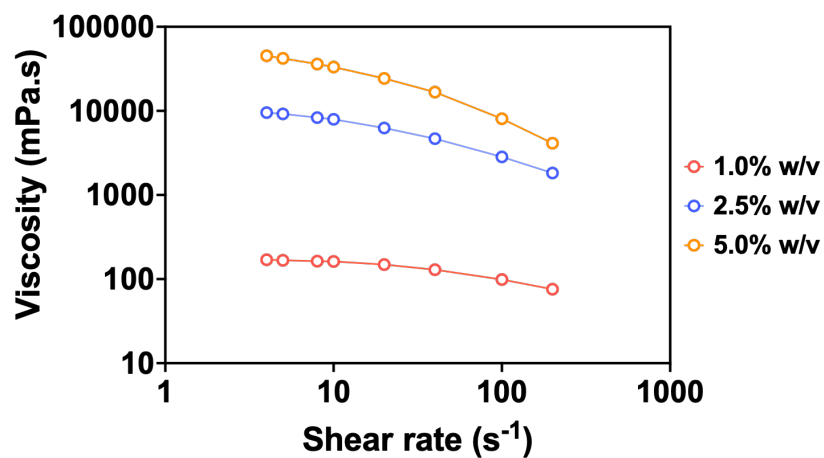


Figure S3.5. Viscosities of sodium alginate solutions with different concentrations.

Table S3.1. Visibility evaluation based on pattern matching scores of samples with different gold amounts under different acrylic resin thicknesses and tube currents.

Acrylic resin thickness (cm)	Tube current (mA)	#1	#2	#3	#4	#5	#6	#7	1.5 mm marker	2.0 mm marker
10	50	80.97	82.04	89.17	78.93	92.47	87.84	97.65	90.54	94.62
10	80	88.76	89.75	93.31	88.46	96.33	93.20	100	95.25	97.27
15	50	42.89	49.17	61.63	43.76	75.74	60.28	83.34	66.66	81.32
15	80	61.70	66.79	74.31	63.44	84.75	72.75	92.49	80.27	89.27
20	50	2.40	1.41	1.73	1.00	32.79	2.08	39.23	25.20	41.62
20	80	16.75	24.95	31.49	4.47	50.76	31.22	58.79	43.21	57.01
20	160	37.60	39.80	51.84	29.25	70.45	46.57	79.57	63.51	72.60

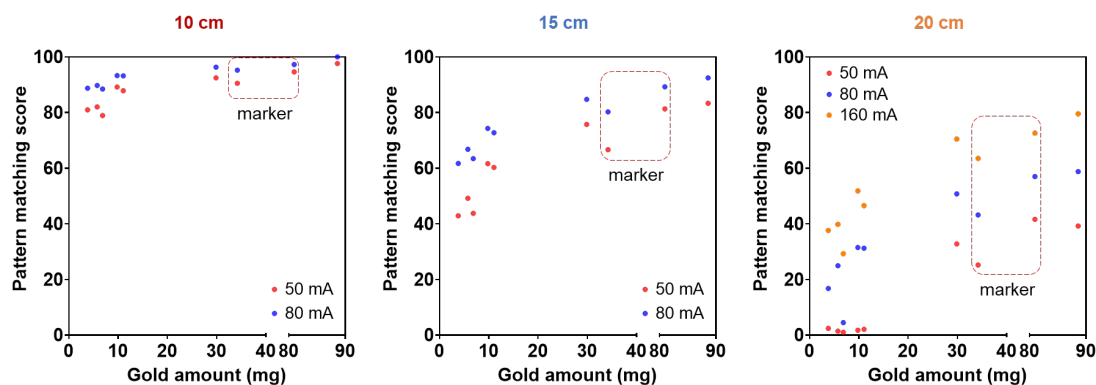


Figure S3.6. Relationship between pattern matching scores of Au NPs and different gold amounts.

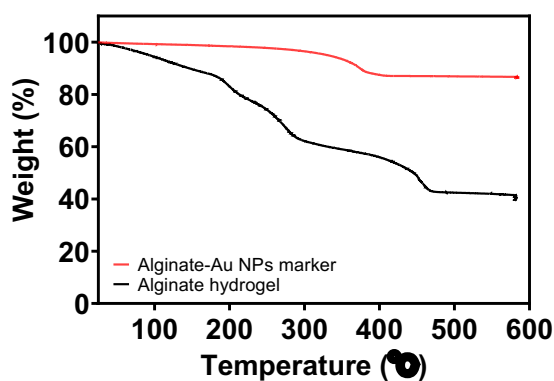


Figure S3.7. TG of Au NPs-based fiducial marker and alginate hydrogel.

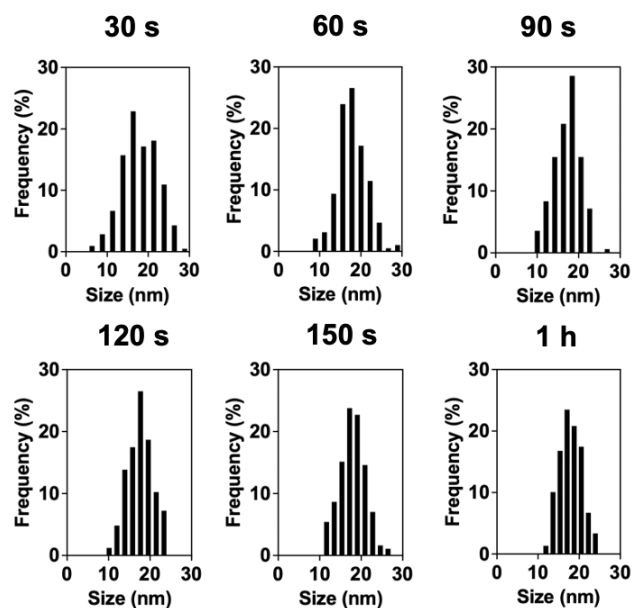


Figure S3.8. Size distributions of Au NPs at different time points.

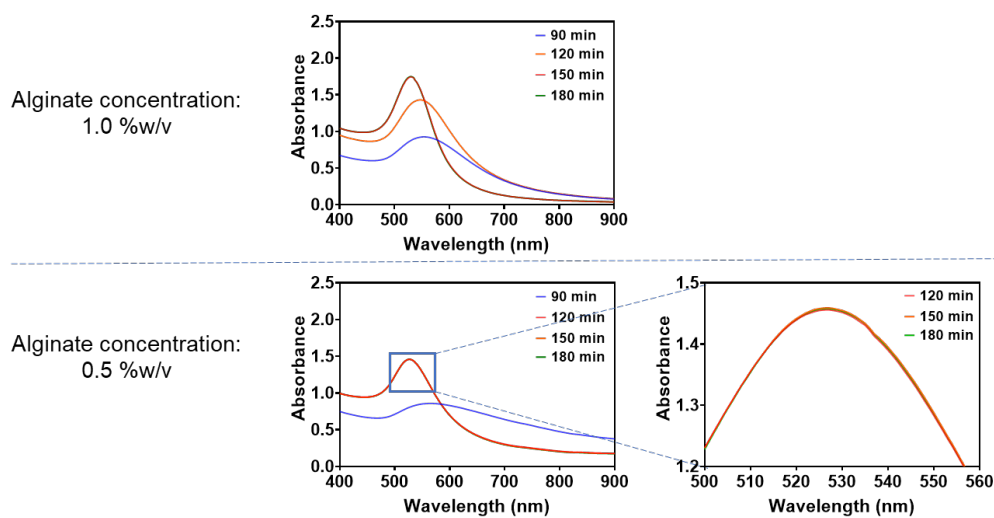


Figure S3.9. UV-Vis spectra of Au NPs synthesized in alginate-only system with different sodium alginate concentrations.

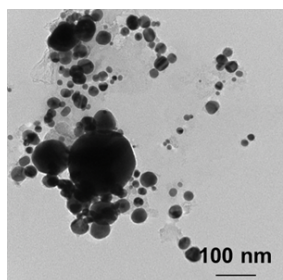


Figure S3.10. TEM of sintered Au NPs processed under 600 °C.

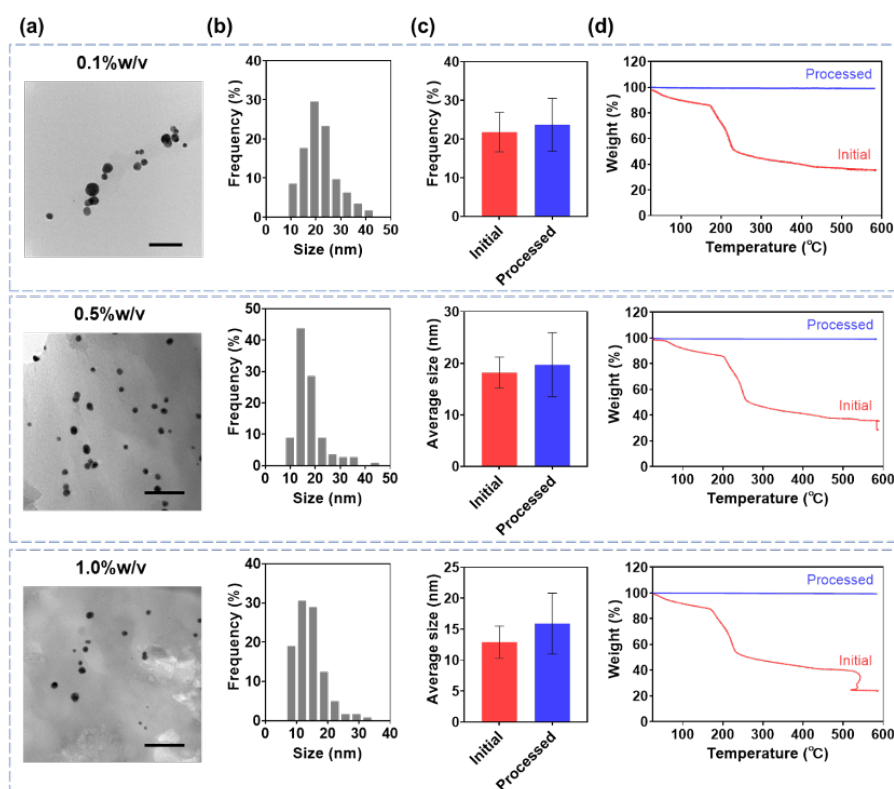


Figure S3.11. (a) TEM and (b) size distributions of alg-Au NPs after thermal treatment. (c) Average sizes and (d) TG of alg-Au NPs before and after thermal treatment.

Experimental steps for TGA:

- Temperature: 25-600 °C
- Temperature rate: 1 °C/min
- Hold time (600 °C): 2 h
- Atmosphere: air

Table S3.2. Visibility evaluation based on pattern matching scores of alg-Au NPs.

Thickness (cm)	Current (mA)	Alg-Au NPs synthesized in different concentrations of alginate aqueous solutions									Gold ball markers	
		0.1% w/v			0.5% w/v			1.0% w/v			1.5 mmφ	2.0 mmφ
		#1	#2	#3	#4	#5	#6	#7	#8	#9		
10	50	76.11	72.72	75.84	75.84	73.34	76.98	74.15	76.67	77.17	71.65	76.07
10	80	78.47	76.48	78.08	78.08	75.93	78.08	76.83	78.41	78.78	75.63	78.02
15	50	64.4	54.57	67.17	67.17	53.3	66.54	55.17	67.47	65.96	51.95	61.03
15	80	72.04	67.02	72.6	72.6	64.45	71.21	67.06	71.92	73.97	60.6	68.88
20	50	35.85	24.1	30.59	30.59	28.01	31.66	19.17	40.15	34.43	23.11	27.48
20	80	50.72	36.76	47.78	47.78	32.03	49.77	37.26	55.76	49.19	31.63	48.49
20	160	60.63	56.87	58.03	58.03	53.97	60.64	50.89	62.63	63.86	47.02	53.20

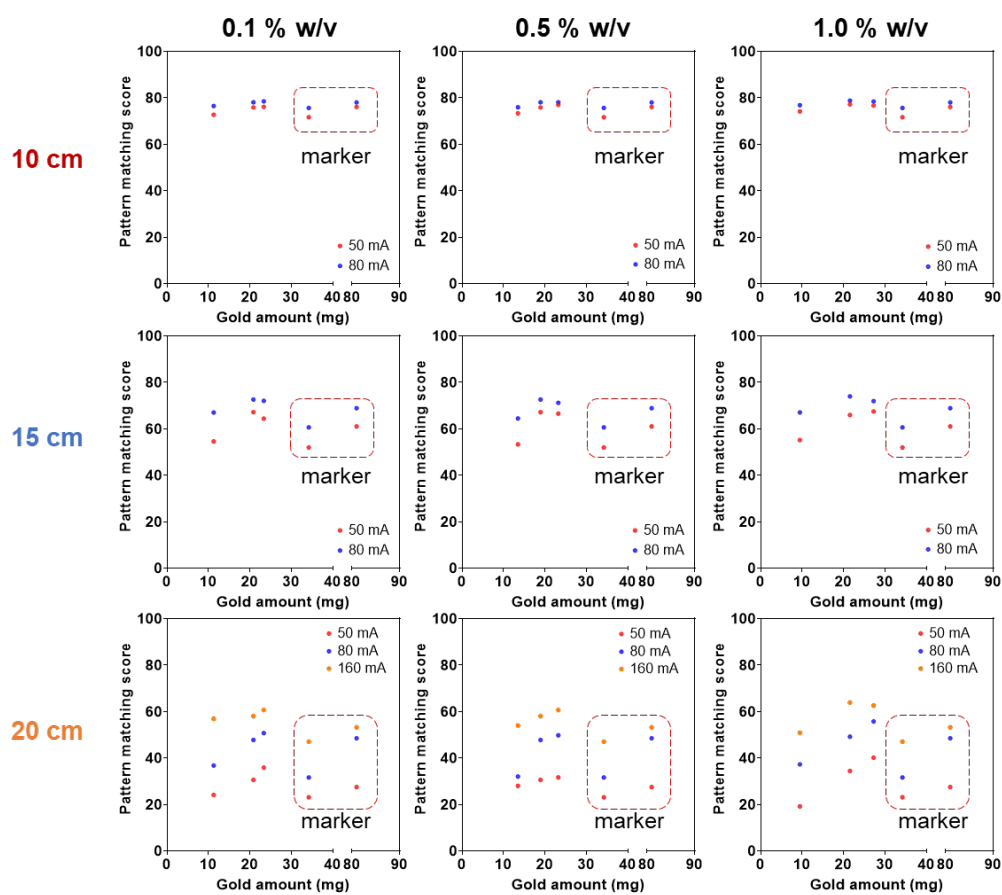


Figure S3.12. Relationship between pattern matching scores of alg-Au NPs and different gold amounts.

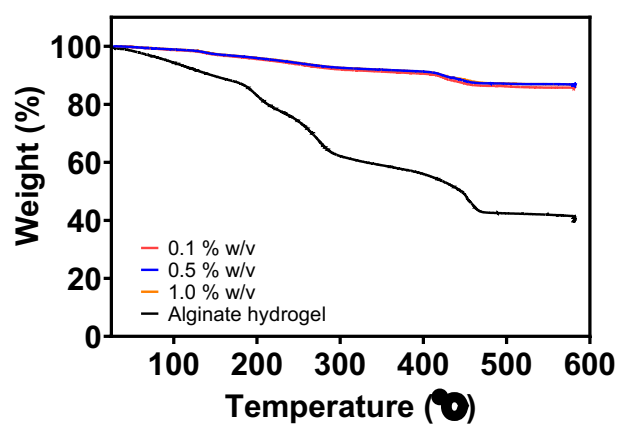


Figure S3.13. TG of alg-Au NPs-based fiducial marker and alginate hydrogel.

3.6. References

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Chapter 4. Ready-To-Use Minimally Invasive Injectable Fiducial Marker Based on the Alginate-Stabilized Gold Nanoparticles and a Body Temperature-Activated *in situ* Gel-Forming System

Abstract

This chapter presents the development of a ready-to-use injectable fiducial marker, GDL/CaCO₃/alg-Au NPs, based on the synthesized alginate stabilized-Au NPs (alg-Au NPs) and a body temperature-activated *in situ* gel-forming system. Gram-scale alg-Au NPs were synthesized in an hour by a green microwave-induced plasma in liquid process (MWPLP). The body temperature-activated *in situ* gel-forming system was developed based on the mild gelation ability of alginate with divalent cations and the temperature-dependent release of calcium source (glucono-delta-lactone (GDL) and CaCO₃). The injectable fiducial marker GDL/CaCO₃/alg-Au NPs could maintain a liquid state at a low temperature for a higher injectability. At body temperature, on the other hand, Ca²⁺ would be released due to the high temperature-activated hydrolysis of GDL and its subsequent reaction with CaCO₃, which will initiate the gelation process of alginate. The injectable fiducial marker can be therefore delivered via injection, and form gel at target site to avoid marker movement or Au NPs leakage *in vivo*. Furthermore, the injectability and imaging ability of GDL/CaCO₃/alg-Au NPs were also examined. Briefly, an injectable fiducial marker GDL/CaCO₃/alg-Au NPs was developed via a green and facile way for IGRT.

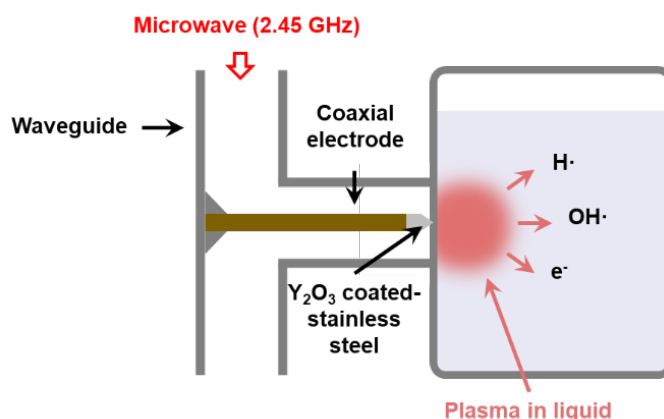
4.1. Introduction

Radiation therapy is a cost-effective and most prevalent modality in cancer treatments[1], as about 50% of patients will receive radiation therapy after diagnosis[2], yet it accounts for only 5% of the total cost for cancer therapy.[3] Radiation therapy involves delivering the therapeutic dose, such as gamma or X-rays, and a stream of particles (alpha, beta, proton, and neutron particles), to target sites to destroy the genetic material of cancer cells.[4] The therapeutic effect of radiation therapy is highly dependent on the coverage of the targets with therapeutic dose and therefore one of the main challenges in radiation therapy is pinpointing the exact location and shape of tumors.[5] However, tumors rarely display a permanent position during irradiation due to breathing motion, peristalsis, and other body motions.[6] A larger three-dimensional planning margin is then required to compensate for the geometric and setup uncertainties, but it also brings increased exposure of normal tissue to radiation. To improve the geometric accuracy and precision of radiation delivery and reduce the doses to the surrounding healthy tissues, image-guided radiation therapy (IGRT) has emerged for optimizing therapeutic efficacy and minimizing treatment toxicity.[7, 8] In IGRT techniques, internal reference points with a fixed position like a skeleton are normally necessary to clearly track the tumors. Fiducial markers are accordingly needed for high tumor positioning accuracy and precise dose delivery in cases that the positions of tumors cannot be correlated with natural reference points. Fiducial markers are generally metal-based solid implants with large physical dimensions. The implantations of these fiducial markers with complicated

insertion procedures are highly invasive, especially for the elderly, and might induce complications, such as pneumothorax,[9, 10] infection,[11, 12] and bleeding. Hence, injectable fiducial markers, including colloidal- and liquid-like markers[13, 14] and a number of commercial markers (Gold Anchor™ Fiducial Markers, PolyMark™ Fiducial Markers, BioXmark®, etc.), are developed as alternatives to current metal-based solid markers. Injectable fiducial markers have distinct advantages like minimally invasive operations and individualized injection volume, representing the cutting edge and a prospective trend of fiducial markers for IGRT.

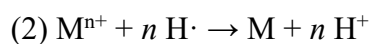
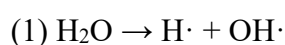
Gold is a kind of remarkable multimodal imaging agent for X-ray imaging,[15] computed tomography (CT),[16, 17] magnetic resonance imaging (MRI),[18] ultrasonic imaging,[19] photoacoustic imaging,[20] owing to its high atomic number, electron density, excellent biocompatibility and stability *in vivo*. Apart from being an imaging agent, gold could be also used as therapeutic agents for photothermal therapy[21-23] and photodynamic therapy[24-26] if necessary. Gold is hence an ideal candidate for fiducial markers for IGRT. For the versatility of possible injection methods, nano-scale gold like gold nanoparticles (Au NPs) are essential. As the most used strategy for the synthesis of Au NPs, chemical reduction methods typically need reducing agents and stabilizing agents for the reduction and the further prevention of aggregation and flocculation between Au NPs. Byproducts might be generated during reactions and cause biological toxicity due to the introduction of reducing and stabilizing agents. In addition, the fixation of Au NPs at objective site is also a prominent

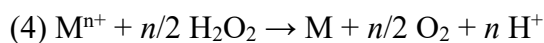
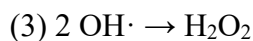
issue because isolated Au NPs would be eliminated from the body through a combination of renal and hepatobiliary pathways.[21] Consequently, an injectable Au NPs-based marker synthesized by a green method with long-term stability *in vivo* is particularly desired.



Scheme 4.1. Schematic illustration of microwave-induced plasma in liquid.

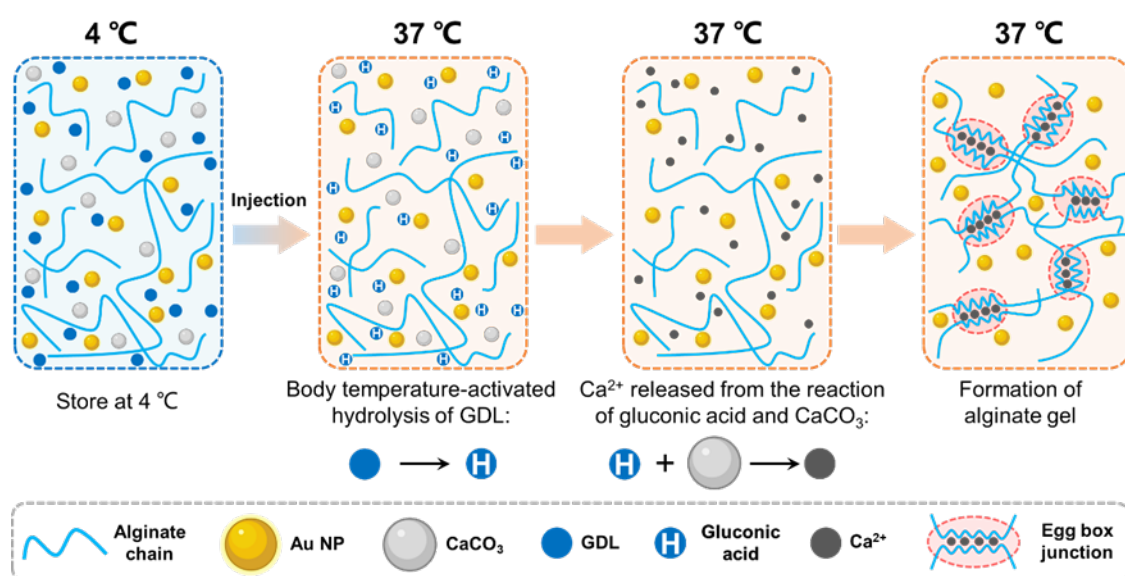
In this chapter, an injectable fiducial marker based on alginate-stabilized Au NPs (alg-Au NPs) was developed. Alg-Au NPs were synthesized by microwave-induced plasma in liquid process (MWPLP). As shown in **Scheme 4.1**, the coaxial electrode works as an antenna to collect microwave energy in the waveguide and eject the energy in the liquid. The plasma discharge generates around the gas (provided by bubbles)/liquid interface. The generated plasma could decompose water molecules to form highly reactive species, including hydrated electrons, hydrogen, and hydroxyl radicals. Reaction equations for this process are summarized in eqs. 1-4:





In these equations, M, H $^\cdot$, and OH $^\cdot$ represent metals, hydrogen radicals, and hydroxyl radicals, respectively. Water is decomposed by plasma to form hydrogen and hydroxyl radicals (eq. 1). Then, hydrogen radicals reduce metal ions (eq. 2). H₂O₂ generated based on the combination of hydroxyl radicals also reduces metal ions (eqs. 3 and 4). MWPLP represents a green alternative in NP synthesis due to its relatively low energy consumption and no need of using toxic reducing agents.[27, 28] For an accelerated reaction and better stability, alginate was introduced in MWPLP. After the addition of alginate, gram-scale alg-Au NPs could be fabricated in an hour and sustained well-dispersed state. Additionally, based on the mild gelation ability of alginate with divalent cations, a body temperature-activated *in situ* gel-forming system was proposed for an easy injection via thin needles and the subsequent fixation of Au NPs at the target site (**Scheme 4.2**). The system GDL/CaCO₃/alg-Au NPs consists of glucono delta lactone (GDL), CaCO₃, and alg-Au NPs. GDL could hydrolyze into gluconic acid and react with CaCO₃, then the generated Ca²⁺ would work as a calcium source for the gelation of alginate. As the hydrolysis of GDL is highly temperature-dependent, temperature can therefore serve as a trigger to regulate the generation of Ca²⁺ and the further gelation process. As illustrated in **Scheme 4.2**, low temperature could limit hydrolysis of GDL and slow the diffusion rate of Ca²⁺, [29] the GDL/CaCO₃/alg-Au NPs can maintain a liquid state during storage. After injection, body temperature promotes the hydrolysis of GDL,

the release of resulting Ca^{2+} would contribute to the formation of alginate gel for the encapsulation and fixation of Au NPs *in vivo*. The injectability of GDL/ CaCO_3 /alg-Au NPs, gelation ability after simulative injection, and the visibility of injected alg-Au NPs were also evaluated. The injectable GDL/ CaCO_3 /alg-Au NPs fulfills the requirements and shows the potential as a fiducial marker for IGRT.



Scheme 4.2. Schematic illustration of a body temperature-activated *in situ* gel-forming system for the fixation of Au NPs at the injection site.

4.2. Experimental Section

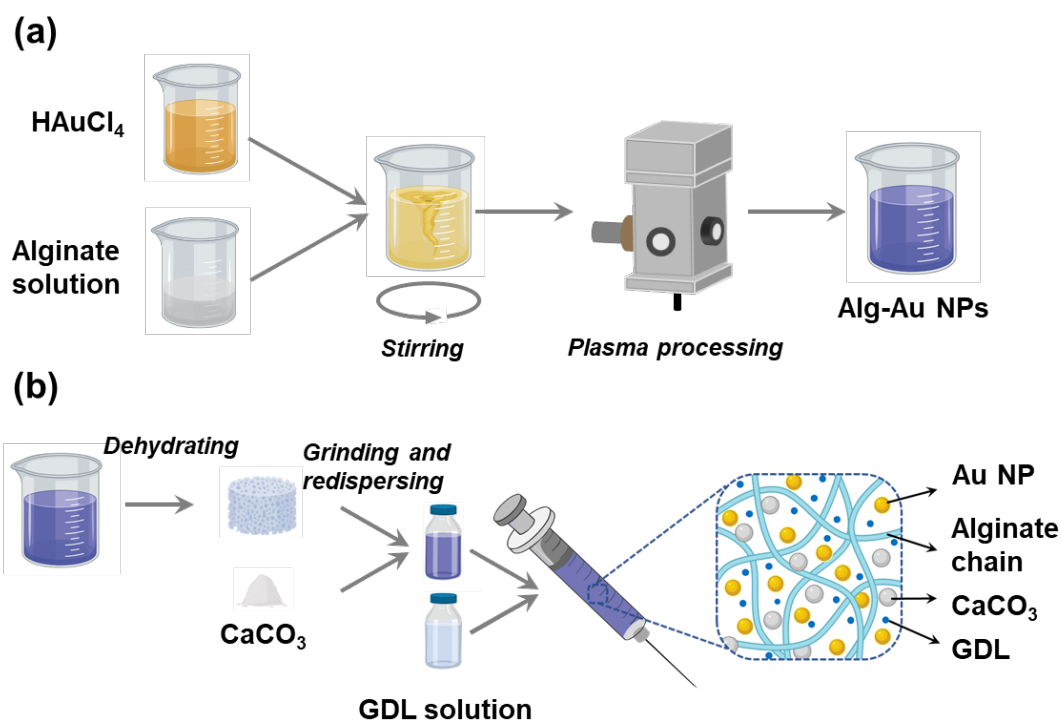
4.2.1. Materials

Tetrachloroauric (III) acid hydrate ($\text{HAuCl}_4 \cdot n\text{H}_2\text{O}$, $n = 3.7$, Kojima, Japan) and sodium alginate (I-8, Kimica, Japan) were used as a precursor and an additive, respectively. 1g $\text{HAuCl}_4 \cdot n\text{H}_2\text{O}$ ($n = 3.7$) was dissolved in 100 mL pure water to obtain a 24.6 mM aqueous

solution of HAuCl_4 . Glucono- δ -lactone (GDL) (Fuso Chemical, Japan) and CaCO_3 (Wako., Japan) were used as a calcium source. All chemicals were used as received. Pure water (Organo/ELGA Purelabo system, $>18.2 \text{ M}\Omega\cdot\text{cm}$) was used to prepare solutions for plasma reaction.

4.2.2. Preparation of Sodium Alginate Stabilized-Au NPs and GDL/ CaCO_3 /alg-Au NPs

The preparation route of alg-Au NPs is illustrated in **Scheme 4.3a**. 0.5 % (w/v) aqueous alginate solution was prepared by dissolving 0.45 g alginate into 90 mL of water. 180 mL of aqueous HAuCl_4 solution (24.6 mM) was added into 90 mL of 0.5 (w/v) aqueous alginate solution. The mixed solution was stirred for 3 h and then transferred into the reaction vessel. The microwave output was kept at 500 W during the reaction and the reaction lasted for 10, 20, 30, 40, 50, 60 min. The GDL/ CaCO_3 /alg-Au NPs was prepared via the process shown in **Scheme 4.3b**. Liquid alg-Au NPs was dried under vacuum and the obtained Au NP powder was grinded with CaCO_3 . The well-mixed powder was redispersed by pure water and mixed with GDL solution. The preparation of GDL solution and following mixing of GDL and powder were operated at a low temperature to avoid unexpected hydrolysis of GDL.



Scheme 4.3. Preparation routes of (a) alg-Au NPs and (b) GDL/ CaCO_3 /alg-Au NPs.

4.2.3. Characterizations

UV-Vis spectra were collected to observe Au NPs formation by using a UV-Vis spectrophotometer (UV-1800, Shimadzu) and a quartz cell with a 1-cm optical path. Samples for UV-Vis spectra measurements were diluted with water with a dilution ratio of 1:1 as the as prepared Au NP dispersions were beyond the detection limit of the UV-Vis spectrophotometer, as shown in **Figure S4.1**. Transmission electron microscopy (TEM, JEOL JEM 2000-FX, at 200 kV) was used to analyze the morphology, size of alg-Au NPs. For TEM sample preparation, alg-Au NPs dispersion was dropped on collodion film-coated copper TEM grids and left for natural drying. The structural details were observed using a scanning

electron microscope (SEM, Miniscope, Hitachi). Thermogravimetric analysis (TG, DTA-60H, Shimadzu) was carried out to evaluate the thermal decomposition behavior of alg-Au NPs and GDL/CaCO₃/alg-Au NPs. Fourier-transform infrared (FTIR) spectroscopy (Nicolet IS10, Thermo Fisher Scientific) was employed to analyze the functional groups of alginate, plasma-processed alginate, alg-Au NPs in the range of 4000-500 cm⁻¹. pH values were collected by pH meter (D-55, Horiba). The viscosities of GDL/CaCO₃/alg and GDL/CaCO₃/alg-Au NPs were measured by a viscometer (DV-I Prime, Brookfield) with shear rate sweeps from 1.0 to 200 s⁻¹ at 4 or 37 °C. To obtain storage and loss moduli of GDL/CaCO₃/alg and GDL/CaCO₃/alg-Au NPs, the rheological measurements were performed on a TA Instruments ARES-G2 rheometer, using a cone and plate geometry with diameter $D = 50$ mm, cone angle $= 0.04$ rad, and gap $d = 1.159$ mm. Time sweep measurements were conducted at a strain amplitude of 1% and angular frequency of 1 rad/s at 4 or 37 °C. Immersion oil (Olympus, Japan) and Evaporation Blocker were utilized to eliminate water evaporation during experiments. The injectability of GDL/CaCO₃/alg and GDL/CaCO₃/alg-Au NPs were tested on an Automatic Vertical Servo Stand (JSV H1000, Algol Instrument Co. Ltd.) with fixed force mode and fixed displacement mode. Briefly, as illustrated in **Figure S4.2**, samples were loaded into a 1-mL syringe with a 21G (inner diameter: 0.51 mm) or 25G (inner diameter: 0.26 mm) injection needle and extruded under a force less than 50 N. The volume percentage of the extruded sample with respect to the total sample was defined as injectability. The injectability under 50 N (surgeons' acceptable maximum force during injection) can be

evaluated via fixed force mode, while the minimum force required for injecting all samples can be measured via fixed displacement mode. To evaluate the X-ray visualization of GDL/CaCO₃/alg-Au NPs, the X-ray visualization test was conducted at the Central Institute of Isotope Science, Hokkaido University. **Figure S4.3** shows the schematic diagram of the X-ray visualization test. The samples were injected into jelly through 25G needles. Then the gelated samples were taken out and irradiated with X-rays generated from an X-ray tube (UD-150-B40, Shimadzu Corporation). The X-rays passed through the samples would be detected by the Flat Panel Detector (PaxScan 3030, Varian Medical System). Besides, acrylic resins that have an X-ray attenuation property close to the human body were placed between samples and the detector to simulate human body,[30] and the visualization ability was evaluated by the pattern matching scores quantified by the detector.

4.3. Results and Discussion

4.3.1. Synthesis of Alg-Au NPs.

As shown in **Scheme 4.3a**, HAuCl₄ was mixed with alginate and then processed by microwave-induced plasma for the synthesis of alg-Au NPs. In **Figure 4.1a**, the formation of alg-Au NPs is confirmed by the UV-Vis spectra, in which a surface plasmon resonance (SPR) peak with maximum absorption wavelength (λ_{max}) centered at around 535 nm could be observed. The absorption intensity reached a maximum after 40 min and then remained steady. In addition, the SPR peaks show an ideal bell shape, which implies that negligible

aggregation occurred and the Au NPs were well dispersed. These results indicate that Au NPs did not aggregate and deposit with the increasing reaction time, validating that the addition of alginate can stabilize Au NPs and prevent the aggregation and deposition caused by inordinate MWPLP processing.

TEM image (**Figure 4.1b**) confirms the formation of alg-Au NPs with a spherical shape. After being processed with MWPLP in alginate solution, the generated Au NPs are uniformly dispersed in the suspensions. The size distribution of alg-Au NPs is approximately 35 nm at different reaction times. Different from the increasing UV-Vis absorption intensity with the increasing reaction time until 40 min, the size distributions are almost constant since 10 min (**Figure 4.1c**). It suggests that the reaction from Au(III) to Au(0) would require approximately 40 min or more to complete, whereas the size of Au NPs would not change after reaching a specific size, implying that the overgrowth of Au NPs could be prevented with presence of alginate, since the cavities formed by alginate chains could function as a template for the growth of Au NPs.[31]

TG was carried out to verify the mass ratio of Au NPs to alginate in alg-Au NPs. Weight loss of alginate is given by the blue line in **Figure 4.1d**, in which 78.8% of total weight lost as a result of dehydration, decomposition of carbonized materials, and further liberation of CO₂. [32] Considering the thermal stability of gold, the weight loss can be attributed to the

removal of alginate. Therefore, alg-Au NPs consists of 61.5 wt% Au NPs and 38.5 wt% of alginate, and the mass ratio of Au NPs to alginate is about 1.6:1.0.

The FTIR spectra of alginate, plasma-processed alginate, and alg-Au NPs were measured to confirm whether the plasma process influenced the chemical structure of alginate. As shown in **Figure 4.1e**, all FTIR spectra show broad peaks at 3500 cm^{-1} attributable to the stretching of hydroxyl groups due to the presence of water. Low-intensity peaks at about 2900 cm^{-1} are associated with the $-\text{CH}_2$ groups. The absorption bands corresponding to the asymmetric and symmetric stretching modes of carboxylate salt groups ($-\text{COONa}$) could be observed at 1610 cm^{-1} and 1417 cm^{-1} . The absorption bands at 1304 cm^{-1} are attributed to the asymmetrical stretching of the C-O-C group, while the vibrations in the range $1100\text{-}1000\text{ cm}^{-1}$ correspond to the glycoside bonds in the polysaccharide (stretching C-O-C). In addition, peaks at 946 cm^{-1} , 890 cm^{-1} , and 818 cm^{-1} are attributed to the C-H vibration of the pyranose (ring of the alginate) group.[33] There is no obvious difference in characteristic absorption bands of alginate, plasma-processed alginate, and alg-Au NPs, demonstrating that the plasma process had an inconsiderable effect on the chemical structure of alginate.

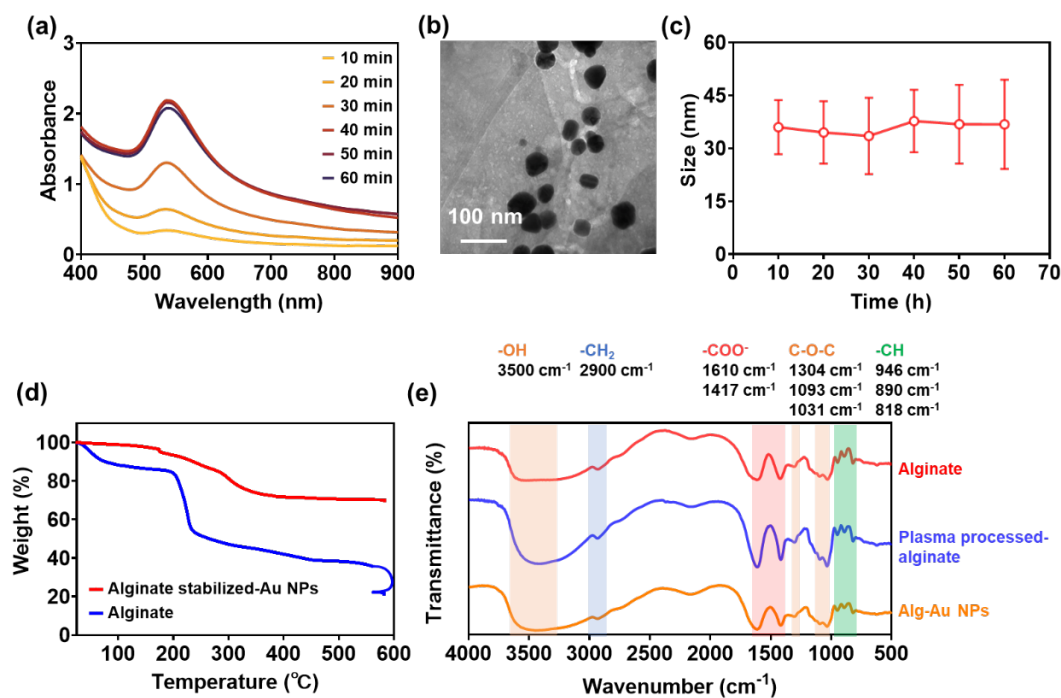


Figure 4.1. (a) UV-Vis spectra of alg-Au NPs for various reaction time, (b) TEM images of alg -Au NPs (60 min), (c) particles size of alg-Au NPs with different reaction time, (d) TG analysis of alg-Au NPs, (e) FTIR of alginate, plasma processed-alginate, and alg-Au NPs.

4.3.2. Body Temperature-Activated *in situ* Gel-Forming System (GDL/CaCO₃/alg)

For the injection of alg-Au NPs via thin needles and their subsequent fixation at the target site, an injectable and body temperature-sensitive GDL/CaCO₃/alg system was developed. In GDL/CaCO₃/alg, the combination of GDL and CaCO₃ serve as a calcium source to induce the gelation of alginate. GDL is recognized as a GRAS (Generally Regarded as Safe) food additive. GDL could hydrolyze into gluconic acid after being dissolved into water, and its hydrolysis rate is highly dependent on the temperature, being slow at ambient temperature and rapid at a higher temperature. This temperature-dependent hydrolysis could result in a variance in gluconic acid concentration between low and high temperatures. Hence,

temperatures can be used as a trigger to control the Ca^{2+} released from GDL/ CaCO_3 and regulate the subsequent gelation behavior of GDL/ CaCO_3 /alg, as illustrated in the **Figure 4.2**. As designed, GDL/ CaCO_3 /alg maintain a liquid-like state at a low storage temperature since the released Ca^{2+} is insufficient for the gelation of alginate, it is therefore easy for the further injection. Conversely, after being injected into body, body temperature activates the hydrolysis of GDL and the release Ca^{2+} , the Ca^{2+} concentration would exceed the lowest Ca^{2+} level required for the alginate gelation, initiating the formation of alginate gel for the fixation of Au NPs.

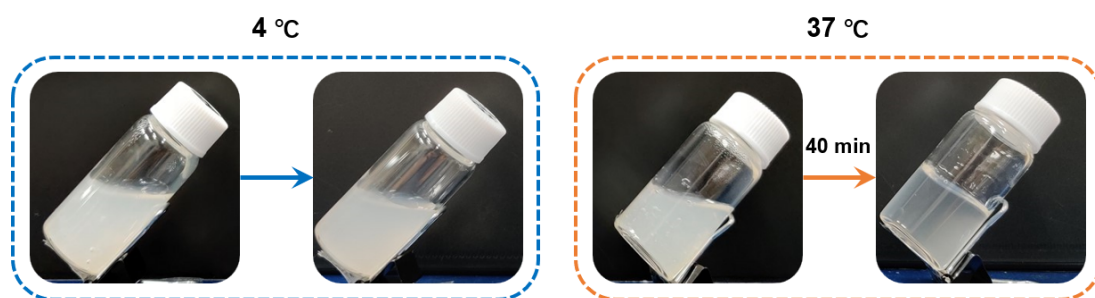


Figure 4.2. Photos showing the gelation behavior of GDL/ CaCO_3 /alg at different temperatures.

The lowest Ca^{2+} concentrations for different concentrations of alginate to gelate were measured and shown in **Table S4.1**. Given the Ca^{2+} release efficiency from the reaction between hydrolyzed GDL and CaCO_3 , gelation performance of GDL/ CaCO_3 /alg with various formulas at different temperatures is shown in **Table S4.2**. High-concentration alginate can increase the loading capacity of Au NPs and prevent their sedimentation for better imaging efficiency, but it is adverse to the injectability of GDL/ CaCO_3 /alg, in which higher extrusion

force are required. Therefore, the optimized formula of 3 % (w/v) alginate, 7 mM CaCO_3 , and 14 mM GDL is selected based on its balanced injectability and temperature-activated liquid-hydrogel transition capability as illustrated in **Figure 4.2**.

To estimate the hydrolysis of GDL, the pH of GDL solution (14 mM) at different temperatures were measured. In **Figure 4.3**, the average pH of GDL solution declined dramatically from 3.83 to 3.14 within the first 8 hours when GDL was kept at 4 °C, revealing that partial hydrolysis of GDL occurred in the first few hours at a low-temperature environment. Thereafter, the pH gradually became steady even after 4 weeks. Based on the pH value of the acid dissociation constant of GDL (3.6), the amount of hydrolyzed GDL can be calculated, and then the hydrolysis rate of GDL is further estimated. The pH of GDL stabilized at 3.03 after 4-weeks storage at 4 °C due to the low hydrolysis limited by the low temperature, and the hydrolysis rate is about 24.3%. Similarly, after being transferred to a 37 °C environment, the pH of GDL solution dropped rapidly from 3.03 to 2.81 at first and then stabilized at about 2.75 after 4 weeks, and the hydrolysis rate increased to 88.3%. Briefly, the temperature-dependent hydrolysis of GDL is confirmed and the variance in hydrolysis rate under different temperatures is the foundation of the liquid-gel transition capability of the GDL/ CaCO_3 /alg system.

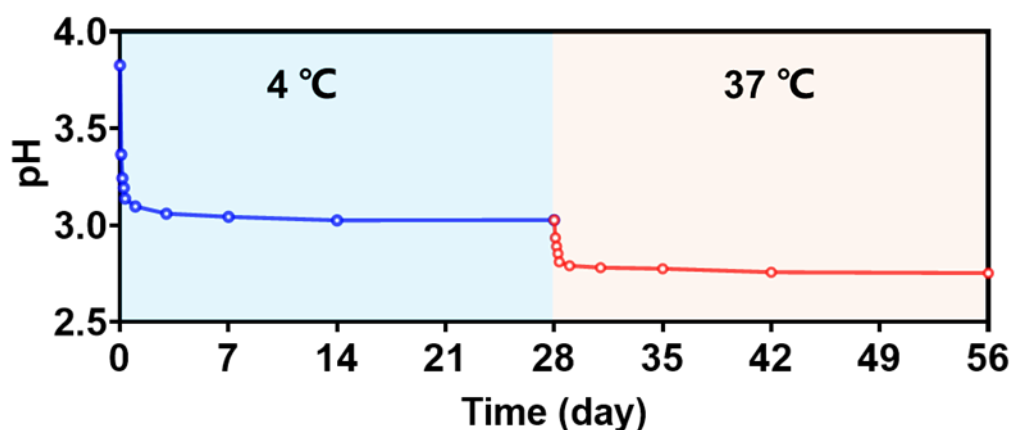


Figure 4.3. pH of GDL solution (14 mM) at different temperatures and storage time.

In GDL/CaCO₃/alg system, the molecular interchange, substantial attractive, cohesive forces between the molecules and alginate chains contribute to its viscosity. After the generation of Ca²⁺, the viscosity of the system would increase along with the ionic crosslinking between alginate chains. The viscosity could therefore reveal these changes and gelation process of the GDL/CaCO₃/alg system. Since lower temperature would increase the cohesive forces while simultaneously reduce the rate of molecular interchange, the viscosities of GDL/CaCO₃/alg at 4 °C are overall larger than those at 37 °C when the storage times are the same. Though the viscosities of GDL/CaCO₃/alg increased at both 4 °C and 37 °C over time (**Figure 4.4a and b**), the increasing rates of viscosities at a shear rate of 1 s⁻¹ are significantly different at different temperatures, as shown in **Figure 4.4c**. The viscosity of GDL/CaCO₃/alg increased 277.8% at 37 °C while the viscosity only increased 89.0% at 4 °C after 4 weeks. As GDL constantly hydrolyzed at 37 °C, the continuous crosslinking between alginate chains would lead to a significant increase in viscosity. In contrast, when GDL just

dissolved in water at 4 °C, only part of GDL turned into gluconic acid and a low degree of crosslinking occurred between alginate chains. These results clearly demonstrate the effect of temperature on the GDL/CaCO₃/alg, i.e., high temperature could promote the crosslinking of alginate chains, while low temperature would limit the hydrolysis of GDL and slow the subsequent gelation process, which ensures the storage stability of GDL/CaCO₃/alg at a low temperature and the gelation at a high temperature. In addition, after being transferred to a 37 °C environment, the viscosity of GDL/CaCO₃/alg increased by 166.7% (from 14288 to 38100 mPa.s) and reached 70.6 % of the maximum viscosity (53976 mPa.s) within 2 hours, suggesting that the body temperature could facilitate the hydrolysis of GDL and the gelation process in a relatively short time.

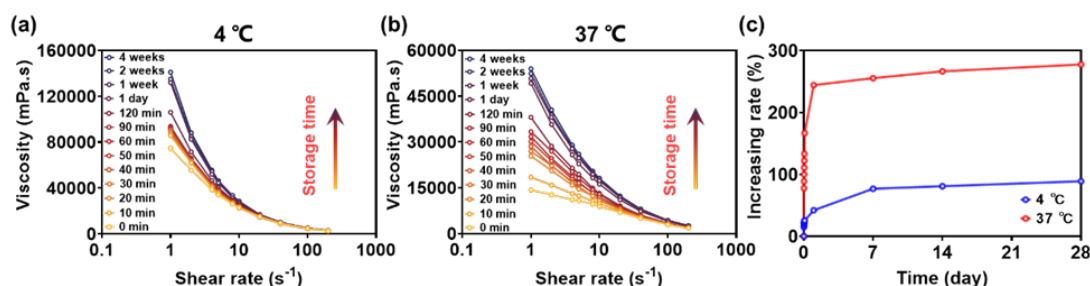


Figure 4.4. Viscosities of GDL/CaCO₃/alg at (a) 4 °C and (b) 37 °C with different storage time, (c) increasing rate of the viscosities of GDL/CaCO₃/alg at 4 °C and 37 °C with different storage time.

The changes in pH of GDL solution and the viscosities of GDL/CaCO₃/alg at different temperatures highlight the feature of temperature-dependent hydrolysis of GDL. To further understand the gelation behavior after a simulative injection into human body and examine

the stability of GDL/CaCO₃/alg at low temperature, the rheological properties of GDL/CaCO₃/alg were studied. For these studies, storage modulus (elastic modulus, G') and loss modulus (viscous modulus, G'') as a function of storage time were analyzed. Firstly, GDL/CaCO₃/alg was stored at 4 °C. As shown in **Figure 4.5a**, when GDL/CaCO₃/alg was just prepared (0 day), it exhibited a typical viscoelastic liquid property: a parallel trend of G' and G'' as a function of time, with G' less than G''. After 1, 2, and 4 weeks, both G' and G'' increased with storage time, suggesting that the partial hydrolysis of GDL and the resulting release of Ca²⁺ promoted the association between alginate chains, which enhanced the elastic behavior of GDL/CaCO₃/alg system. However, G'' were still dominant compared to G', indicating the system was still liquid-like. Therefore, storing GDL/CaCO₃/alg at a low temperature is an effective way to maintain its liquid state and ensure its subsequent injectability. In contrast, in **Figure 4.5b**, when GDL/CaCO₃/alg was kept at 37 °C, G' became larger than G'' after only 1 day of storage, showing a gel-like behavior. To study the gelation process of GDL/CaCO₃/alg, the system was transferred from a refrigerator (4 °C) to the rheometer (37 °C) to simulate the temperature change after injection, and a time sweep was then conducted. The gel formation process of GDL/CaCO₃/alg is confirmed by its rheological properties (**Figure 4.5c**), which shows a gradual transition from liquid-like (G' < G'') to solid-like behavior (G' > G'') over time. At the first stage (0-28 min), G'' of GDL/CaCO₃/alg was smaller than G', exhibiting a liquid state. The gel point (28 min), which is defined as a point of sol-gel transition (G' = G''), was observed and represented the transition from liquid to gel.

After passing the gel point (> 28 min), G' and G'' continued growing and G' became dominant, showing a gel-like state and an enhanced mechanical strength. Briefly, from the rheological properties, the GDL/ CaCO_3 /alg can work as designed: when GDL/ CaCO_3 /alg is stored at low temperature, it could keep liquid state; after being injected into an environment with body temperature, the hydrolysis of GDL could be activated by the increased temperature, and the accelerated release of Ca^{2+} would promote the gelation of alginate in 28 mins.

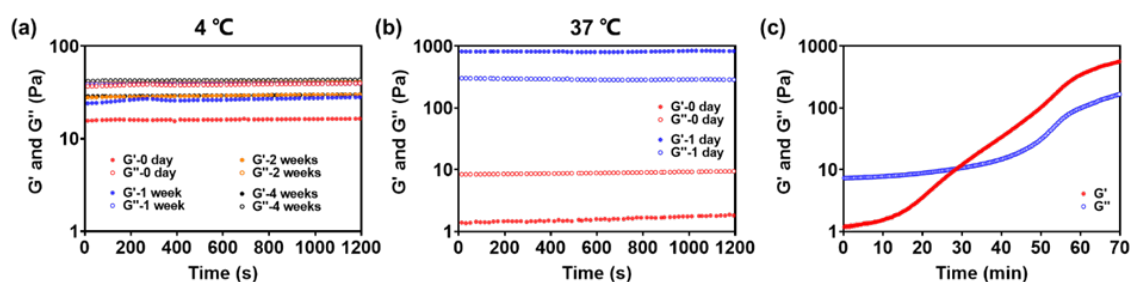


Figure 4.5. Rheological characterization of GDL/ CaCO_3 /alg at (a) 4 °C and (b) 37 °C, (c) changing rheology of GDL/ CaCO_3 /alg after being stored at 37 °C.

The structural details of the gelated GDL/ CaCO_3 /alg were examined by SEM. As shown in **Figure 4.6**, the inner structure of GDL/ CaCO_3 /alg gel could be observed. The gel consists of firm and uniform fibers, and has a highly porous structure with interconnected pores.

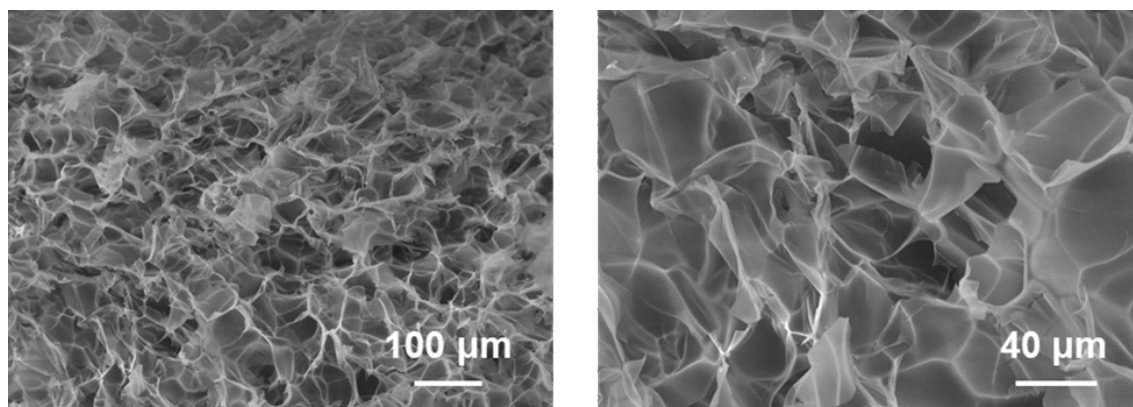


Figure 4.6. SEM images of GDL/CaCO₃/alg gel formed at 37 °C

4.3.3. Temperature-Activated Gelation Performance of GDL/CaCO₃/alg-Au NPs

The body temperature-activated gelation capability of GDL/CaCO₃/alg has been demonstrated, implying its potential for the delivery and fixation of Au NPs *in vivo*. In this section, alg-Au NPs were combined with GDL/CaCO₃ to verify whether the presence of Au NPs would influence the injectable body temperature-activated gel-forming system. The viscosities of GDL/CaCO₃/alg-Au NPs are higher than that of GDL/CaCO₃/alg when the temperature and storage time are the same. **Figure 4.7a and b** shows that the viscosities of GDL/CaCO₃/alg-Au NPs at 4 °C are overall larger than that at 37 °C, which are similar to GDL/CaCO₃/alg. And the viscosities of GDL/CaCO₃/alg-Au NPs also increased at both 4 °C and 37 °C over time. On the other hand, as indicated in **Figure 4.7c**, the viscosity of GDL/CaCO₃/alg-Au NPs increased 280.4% at 37 °C, while the viscosity only increased 60.2% at 4 °C after 4 weeks. This further demonstrates different crosslinking behaviors of GDL/CaCO₃/alg at different temperatures and the addition of Au NPs has a negligible effect

on gelation ability the GDL/CaCO₃/alg system. Likewise, in a 37 °C environment, the viscosity of GDL/CaCO₃/alg-Au NPs increased by 185.0% (from 32864 to 93664 mPa.s) and reached 74.9 % of the maximum viscosity (125000 mPa.s), indicating that the body temperature could still facilitate the hydrolysis of GDL and the gelation process in a short time with the presence of Au NPs.

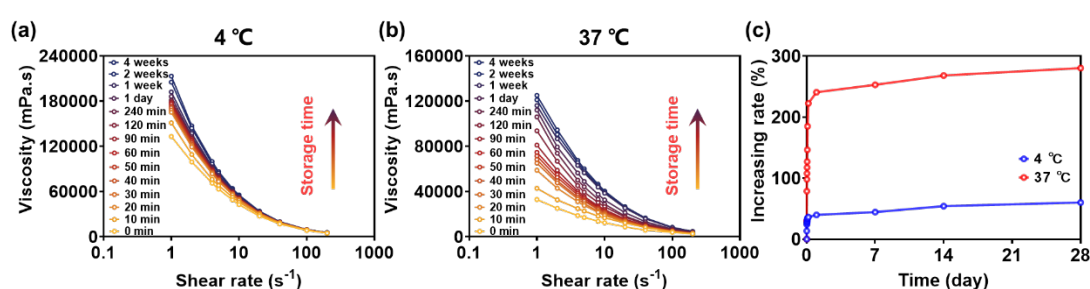


Figure 4.7. Viscosities of GDL/CaCO₃/alg-Au NPs at (a) 4 °C and (b) 37 °C with different storage time, (c) increasing rate of viscosities of GDL/CaCO₃/alg-Au NPs at 4 °C and 37 °C with different storage time.

Same rheological measurements were conducted to assess the stability of GDL/CaCO₃/alg with the addition of Au NPs at the storage temperature and its gelation behavior at body temperature. As shown in **Figure 4.8a**, after being stored at 4 °C for 1, 2, and 4 weeks, GDL/CaCO₃/alg-Au NPs still had typical liquid-like rheological properties as it was just prepared, in which G' was smaller than G'' with a parallel trend, displaying an excellent storage stability. Conversely, in **Figure 4.8b**, when GDL/CaCO₃/alg-Au NPs was stored at 37 °C, it showed a gel-like behavior with G' larger than G'' after only 1 day. The sol-gel transition of GDL/CaCO₃/alg-Au NPs was also investigated by transferring it from a

storage temperature to a body temperature environment, as shown in **Figure 4.8c**. Similar to GDL/CaCO₃/alg, the gel formation process of GDL/CaCO₃/alg-Au NPs also consists of three stages: 1) liquid state (0-32 min) with G' smaller than G'' ; 2) gel point (32 min) with G' equals to G'' ; and 3) gel state (after 32 min) with G' larger than G'' . As illustrated in **Figure 4.9**, though the presence of Au NPs slightly delays the sol-gel transition of GDL/CaCO₃/alg-Au NPs, the system could still work as designed to maintain liquid state at low temperature for easier injection and turn into gel to fix Au NPs for a high positioning accuracy after injection, making GDL/CaCO₃/alg-Au NPs a suitable injectable marker for IGRT.

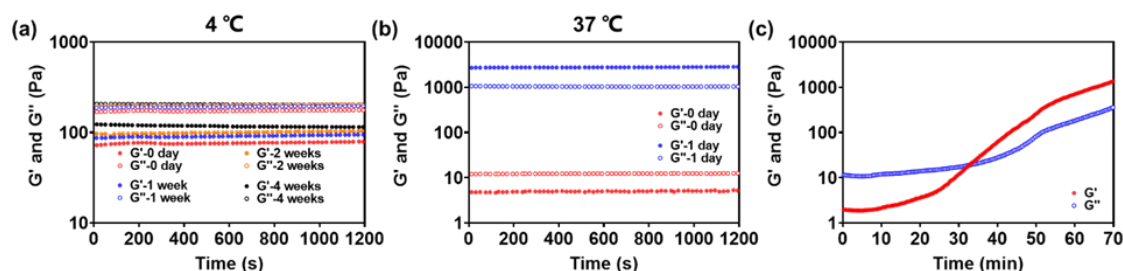


Figure 4.8. Rheological characterization of GDL/CaCO₃/alg-Au NPs at (a) 4 °C and (b) 37 °C, (c) changing rheology of GDL/CaCO₃/alg-Au NPs after being stored at 37 °C.

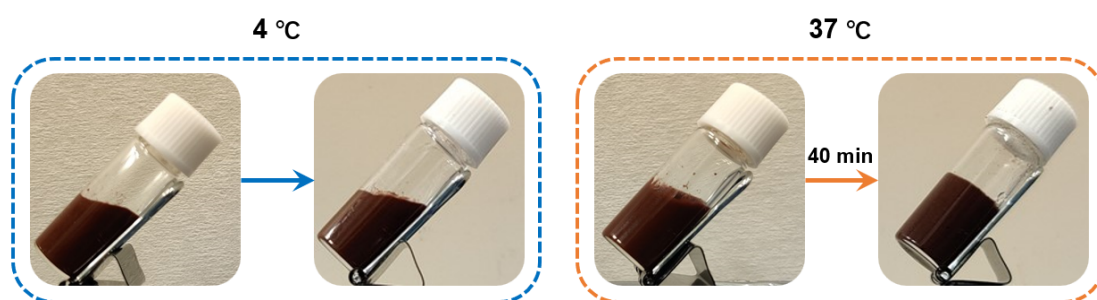


Figure 4.9. Photos showing the gelation behavior of GDL/CaCO₃/alg-Au NPs at different temperatures.

Figure 4.10 shows the SEM of GDL/CaCO₃/alg-Au NPs. Compared to GDL/CaCO₃/alg (**Figure 4.6**), subtle differences in terms of pore size and degree of compactness could be observed, but GDL/CaCO₃/alg-Au NPs has a similar porous structure with uniform fibers and interconnected pores.

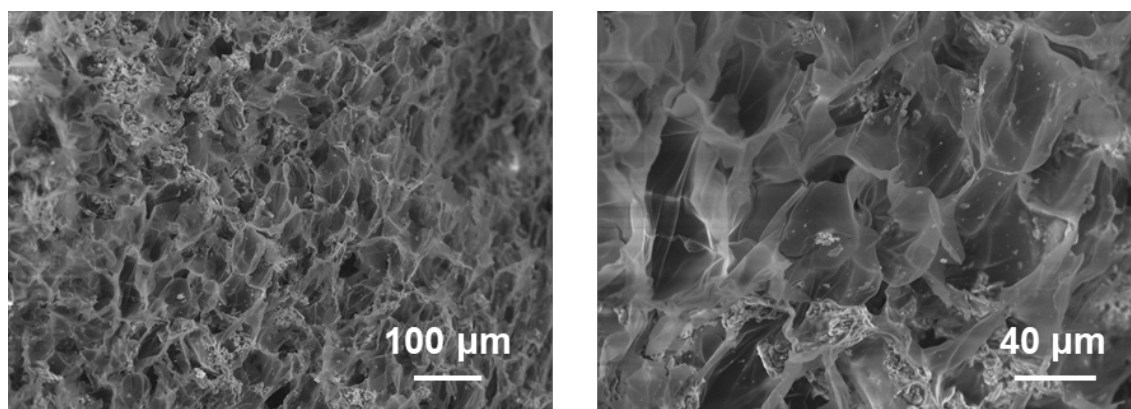


Figure 4.10. SEM of GDL/CaCO₃/alg-Au NPs gel formed at 37 °C.

4.3.4. Injectability of GDL/CaCO₃/alg-Au NPs

Figure 4.11 shows the injectability of GDL/CaCO₃/alg and GDL/CaCO₃/alg-Au NPs through 21G and 25G needles. Firstly, the amounts of GDL/CaCO₃/alg and GDL/CaCO₃/alg-Au NPs that could be injected via a fixed extrusion force were measured via a fixed force mode, as shown in **Figure S4.2a**. In **Figure 4.11a**, an extrusion force of 50 N, which is the maximum force that acceptable for surgeons during operation, was applied on the syringe, 100% GDL/CaCO₃/alg and GDL/CaCO₃/alg-Au NPs could be injected through both 21G (inner diameter: 0.51 mm) and 25G (inner diameter: 0.26 mm) needle, exhibiting an excellent injectability and a minimally invasive property. To quantitatively characterize the injectability

of GDL/CaCO₃/alg and GDL/CaCO₃/alg-Au NPs, the minimum extrusion forces (**Figure 3.11b**) required for 100% injectability were measured via a fixed displacement mode, as shown in **Figure S4.2b**. Extrusion curves are normally characterized by a very rapid increase of the force followed by a plateau. As indicated in **Figure 4.11b**, 7.8 N and 8.1 N are required for 100% injection of GDL/CaCO₃/alg and GDL/CaCO₃/alg-Au NPs with 21G needles, while 21.8 N and 28.1 N are necessary for 25G needles. During injection, the force applied to a syringe plunger would be dissipated in three ways: (a) overcoming the resistance force of the syringe plunger; (b) imparting kinetic energy to the liquid; and (c) forcing the liquid through the needle.[34, 35] A larger force is required for passing through a narrow needle, so the minimum extrusion force for a 25G needle is overall higher than that for a 21G needle. Meanwhile, the addition of Au NPs would cause a higher kinetic energy and driving force of the whole system, which led to an increasing extrusion force for GDL/CaCO₃/alg-Au NPs to pass through needles. Benefitting from the high viscosity of GDL/CaCO₃/alg, Au NPs cannot overcome the lateral drag force from GDL/CaCO₃/alg and, therefore, these NPs will not entirely deposit as they would in less viscous solution,[36] ensuring the injectability and stability of GDL/CaCO₃/alg-Au NPs after long-term storage. Notably, even the highest extrusion force (28.1 N) is smaller than 50 N, and such a relatively small force provides considerate scope for further optimization of the formulation and improvement in the imaging performance of GDL/CaCO₃/alg-Au NPs.

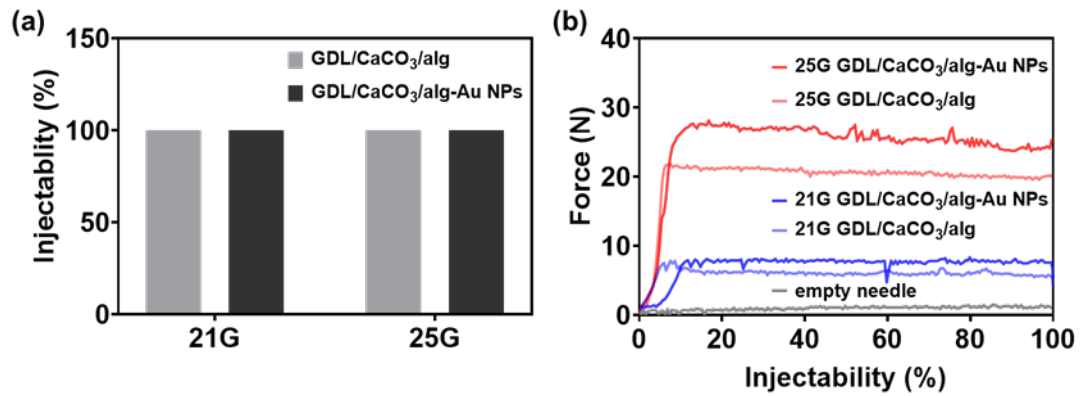


Figure 4.11. (a) Injectability of GDL/CaCO₃/alg and GDL/CaCO₃/alg-Au NPs with 21G and 25G needles under 50 N, (b) Minimum force required for 100 % injection of GDL/CaCO₃/alg and GDL/CaCO₃/alg-Au NPs by 21G and 25G needles.

4.3.5. Imaging Performance of GDL/CaCO₃/alg-Au NPs

Figure 4.12 shows images obtained from an X-ray visualization test and **Table 4.1** shows the results of the pattern matching score calculated based on X-ray visualization images. A pattern matching score is regarded as a quantitative measurement of the visibility of an object in relation to its surroundings. The pattern matching score ranging from 1 to 100 is calculated by the correlation coefficient. A pattern matching score over 30 indicates that the marker could be recognized correctly by the detector with high tracking accuracy and is considered to have sufficient visibility for motion tracking during IGRT. Under the same resin thickness, a higher score indicates better visibility. As shown in **Figure 4.12**, the pattern matching scores decreased with increasing acrylic resin thickness, suggesting the human body also causes the attenuation of X-ray. The higher pattern matching score under thicker acrylic resin suggests that the marker would have better imaging performance deep inside the body.

When the acrylic resin increased to 15 cm, GDL/CaCO₃/alg-Au NPs become hard to identify. The gold amounts are calculated by TG analysis and the relationship of pattern matching score and the gold amounts is shown in **Figure 4.13**. A linear relationship between pattern matching score and gold amounts could be observed. Compared to the gold markers, the visibilities of GDL/CaCO₃/alg-Au NPs are inferior due to the less amount of gold. However, based on the pattern matching score of gold markers and the fit lines of GDL/CaCO₃/alg-Au NPs, it implies that a similar pattern matching score could be obtained with fewer gold amounts. In addition, the imaging performance of GDL/CaCO₃/alg-Au NPs deep inside the body can be further enhanced by strategies that can increase the ratio of Au NPs to alginate, such as removing excess alginate through thermal process, which has been validated in Chapter 3. Considering the overall simplicity of the GDL/CaCO₃/alg-Au NPs system and its acceptable imaging performance, this chapter does not adopt these strategies to complicate the development of Au NP-based fiducial markers.

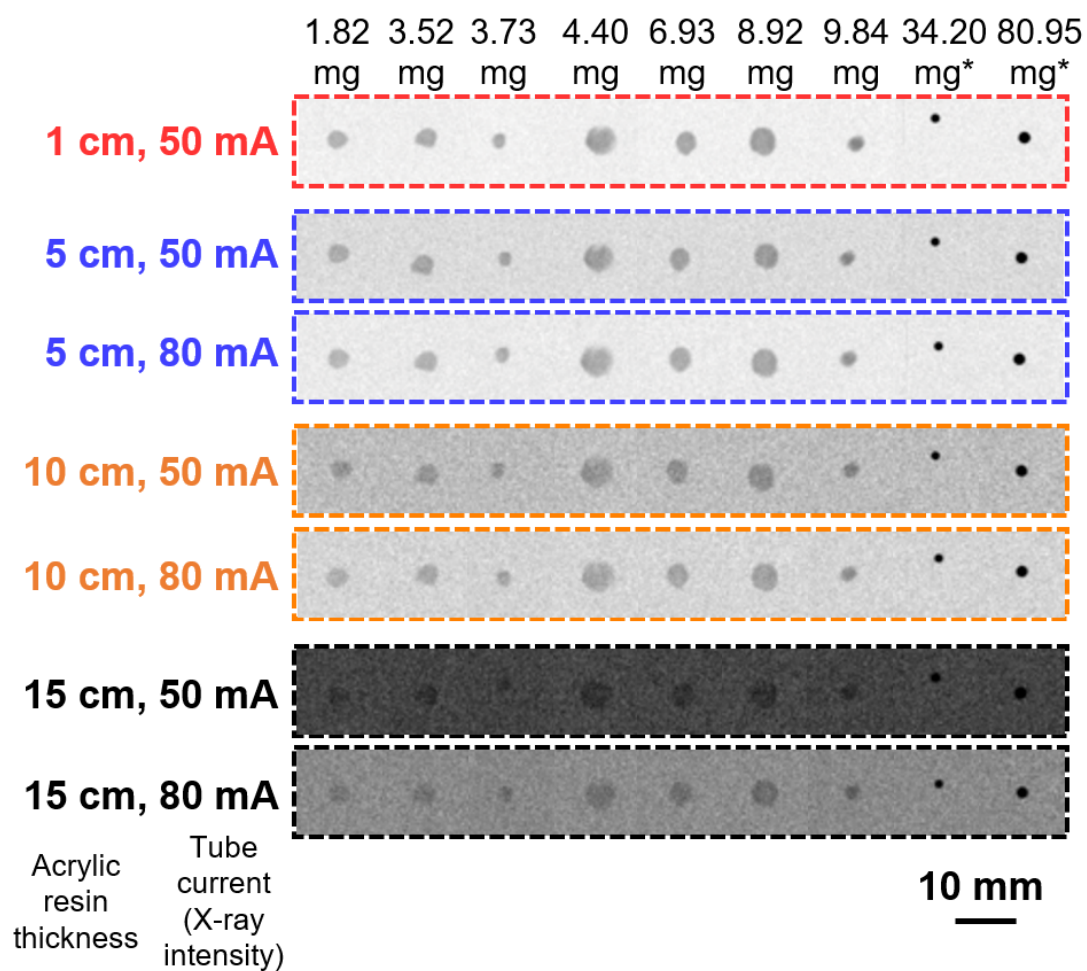


Figure 4.12. X-ray visualization image of gold markers and GDL/CaCO₃/alg-Au NPs with different gold amounts. *Mass of 1.5-mm gold marker (34.2 mg) and 2.0-mm gold marker (80.95 mg)

Table 4.1. Visibility evaluation based on pattern matching scores with different gold amounts.

Tube current (mA)	Acrylic resin thickness (cm)	1.82 mg	3.52 mg	3.73 mg	4.40 mg	6.93 mg	8.92 mg	9.84 mg	34.2 mg*	80.95 mg*
50	1	88.35	91.20	92.94	93.01	94.27	94.65	96.16	99.00	99.53
50	5	70.71	72.70	75.84	78.59	82.22	83.26	87.52	97.38	98.52
80	5	80.84	82.93	86.12	86.88	89.41	89.73	92.39	98.04	99.14
50	10	33.98	39.96	40.90	43.68	50.43	53.29	62.74	90.04	94.45
80	10	43.63	52.32	56.44	59.60	63.52	65.23	72.46	93.63	96.46

* Mass of 1.5-mm spherical gold marker (34.2 mg) and 2.0-mm gold marker (80.95 mg)

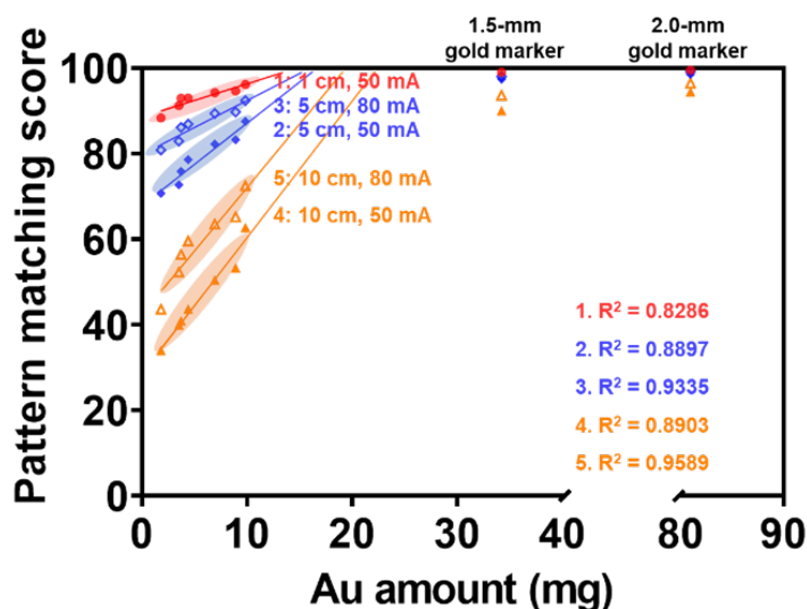


Figure 4.13. Relationship between pattern matching scores and different gold amounts.

4.4. Conclusions

An injectable fiducial marker based on alg-Au NPs and a body temperature-activated *in situ* gel-forming system has been developed. Gram-scale Au NPs were prepared by a green method MWPLP in an hour. The addition of alginate could effectively stabilize a large

amount of Au NPs and prevent their aggregation via the cavities formed by alginate chains. The body temperature-activated *in situ* gel-forming system GDL/CaCO₃/alg was designed based on the mild gelation ability of alginate with divalent cations and the temperature-dependent release of calcium source. GDL/CaCO₃/alg-Au NPs could maintain a liquid state when stored at low temperature due to the slow hydrolysis of GDL and release of Ca²⁺. The hydrolysis of GDL could be activated and accelerated by body temperature after injection, resulting in the *in situ* formation of alginate gel. This nature allows for excellent injectability and stability of GDL/CaCO₃/alg-Au NPs at the storage temperature, and the capability of transforming into gel to fix Au NPs at the target site for an improved geometric accuracy, making it a ready-to-use injectable fiducial marker that is convenient for surgeons and minimally invasive for patients.

4.5. Supporting Information

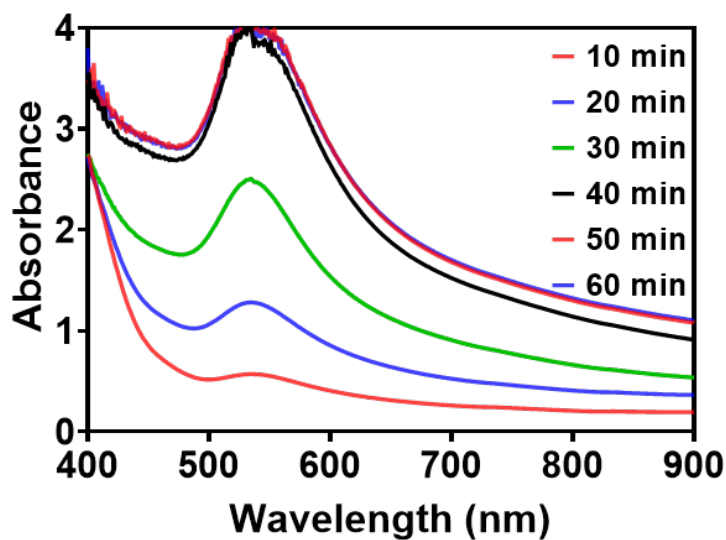


Figure S4.1. UV-Vis spectra of alg-Au NPs with different reaction time.

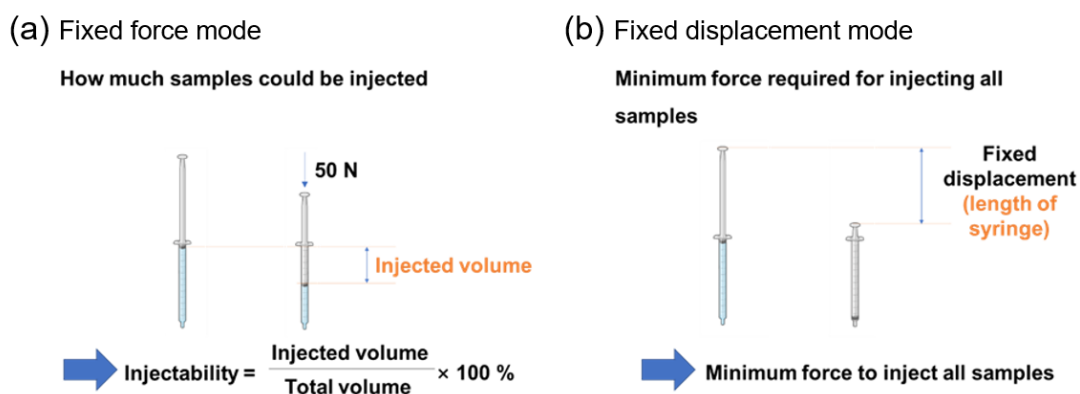


Figure S4.2. Illustration of injectability test. (a) Fixed force mode to evaluate how much sample could be injected under 50 N, a force equals to that of surgeons' hand during injection. (b) Fixed displacement mode to evaluate the minimum force required for injecting all samples.

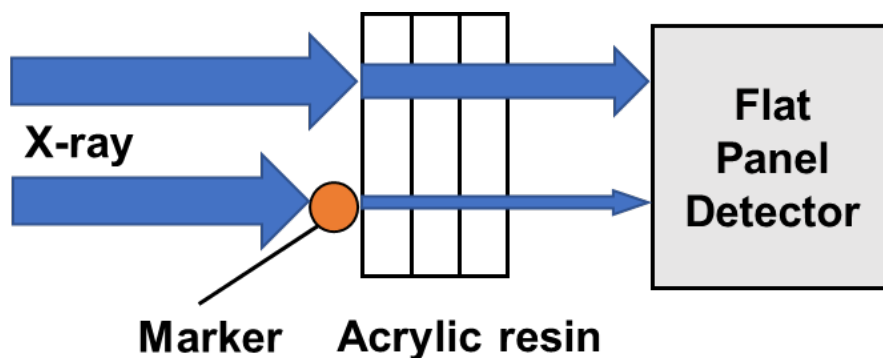


Figure S4.3. Schematic diagram of the X-ray transmission test.

Table. S4.1. Lowest Ca^{2+} concentrations for different concentrations of alginate to gelate

A: alginate solution (4, 6, 8 % w/v)

B: CaCl_2 solution (2, 4, 6, 8, 10, 12, 14, 16 mM)

5 mL **A** + 5 mL **B**

Ca ²⁺ concentration	Alginate concentration		
	2 % w/v	3 % w/v	4 % w/v
1 mM	not gelled	not gelled	not gelled
2 mM	not gelled	not gelled	not gelled
3 mM	not gelled	not gelled	slightly gelled, weak
4 mM	not gelled	slightly gelled, weak	gelled
5 mM	slightly gelled, weak	gelled	gelled
6 mM	slightly gelled, weak	gelled	gelled
7 mM	gelled	gelled	gelled
8 mM	gelled	gelled	gelled

Table. S4.2. Gelation performance of GDL/CaCO₃/alg with various formulas at different temperatures

A: alginate solution (4, 6, 8 % w/v) contained CaCO₃ (6, 8, 10, 12, 14 mM)

B: GDL solution (12, 16, 20, 24, 28 mM)

Molar ratio of CaCO₃ to GDL is 1:2

5 mL **A** + 5 mL **B**

Alginate concentration	CaCO ₃ concentration	GDL concentration	4 °C	37 °C
2 % (w/v)	5 mM	10 mM	not gelled	not gelled
	6 mM	12 mM	not gelled	not gelled
	7 mM	14 mM	not gelled	not gelled
3 % (w/v)	5 mM	10 mM	not gelled	not gelled
	6 mM	12 mM	not gelled	slightly gelled, weak
	7 mM	14 mM	not gelled	gelled
4 % (w/v)	3 mM	6 mM	not gelled	slightly gelled, weak
	4 mM	8 mM	slightly gelled, weak	gelled
	5 mM	10 mM	gelled	gelled

4.6. References

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Chapter 5. Conclusions

This study explores two strategies for the green, effective, and biocompatible synthesis of Au NPs and evaluates the potential of the synthesized Au NPs as injectable and minimally invasive fiducial markers. A ready-to-use injectable fiducial marker is developed for IGRT based on the synthesized Au NPs and a body temperature-activated *in situ* gel-forming system. The key results and conclusions are summarized as follows:

Chapter 2 describes the synthesis of alg-Au NPs using MWPLP. Alginate was introduced for (1) accelerating the reaction rate, (2) preventing aggregation and deposition due to long time discharge in MWPLP, and (3) providing long-term stability. The size of synthesized Au NPs can be controlled by regulating the alginate concentrations. An abnormal size change (from large to small) of Au NPs during their formation process was observed and a possible mechanism based on dynamical and thermodynamical instability is proposed. The strategy of drying and redispersion of Au NPs in alginate solution had an imperceptible effect on the Au NPs, suggesting this strategy has the potential as an effective technique for the long-term storage of Au NPs and other metal NPs. In addition, the synthesis of alg-Au NPs without using toxic reducing or stabilizing agents renders them appropriate for biomedical applications.

Chapter 3 presents the Au NPs synthesized via ethanol-only and ethanol/alginate systems. Their injectability and imaging capabilities as fiducial markers were evaluated. The injection medium viscosity, Au NPs concentration, and needle gauge were shown to be the most important factors influencing the injectability of Au NPs, whereas gold amount and particle size dominated their imaging performance. Compared to clinically used gold ball markers (1.5 or 2.0 mm ϕ), Au NPs prepared in the ethanol-only system had an improved injectability (through 18G and 21G needles) and an almost identical imaging capability. The novel reducing system based on the combination of ethanol and alginate. The addition of alginate can significantly improve the reaction efficiency, control the size of synthesized Au NPs, and prevent the aggregation between Au NPs. Due to the size control during synthesis, the alg-Au NPs with smaller sizes (14.1 to 21.8 nm) exhibited a superior injectability, up to 100% even through 25G needles with extrusion force less than 17 N. A better imaging capability of alg-Au NPs was also verified in the X-ray visualization test. These results demonstrate the potential of alg-Au NPs as injectable and minimally invasive fiducial markers.

Chapter 4 focuses on the development of a ready-to-use injectable fiducial marker, GDL/CaCO₃/alg-Au NPs, based on the synthesized alg-Au NPs and a body temperature-activated *in situ* gel-forming system. The body temperature-activated *in situ* gel-forming system GDL/CaCO₃/alg was designed based on the mild gelation ability of alginate with divalent cations and the temperature-dependent release of calcium source. GDL/CaCO₃/alg-Au NPs could maintain a liquid state when stored at a low temperature due to the slow

hydrolysis of GDL and release of Ca^{2+} . The hydrolysis of GDL could be activated and accelerated by body temperature after injection, resulting in the *in situ* formation of alginate gel. This nature allows for excellent injectability and stability of GDL/ CaCO_3 /alg-Au NPs at the storage temperature, and the capability of transforming into gel to fix Au NPs at the target site for an improved geometric accuracy, making it a ready-to-use injectable fiducial marker that is convenient for surgeons and minimally invasive for patients.

As this injectable fiducial marker is designed to be used in humans' body during IGRT, attention should also be paid to its biological evaluation in subsequent work:

- *in vitro* evaluations: cytotoxicity, degradation, cellular uptake, and apoptosis
- *in vivo* evaluations: injectability, acute toxicity, stability, distribution, metabolism, imaging performance
- other potential functions: radiosensitization, photothermal conversion

List of Publications

1. Liu, H., Ikeda, K., Mai, NT., Sato, S., Matsuda, N., Tsukamoto, H., Tokumaga, T., Yonezawa, T. Alginate-Stabilized Gold Nanoparticles Prepared Using the Microwave-Induced Plasma-in-Liquid Process with Long-Term Storage Stability for Potential Biomedical Applications[J]. ACS omega, 2022, 7(7): 6238-6247.
2. Ikeda K.#, Liu H.#, Miyamoto N., Mai, NT., Shirato, H., Yonezawa, T. Preparation of Biopex-Supported Gold Nanoparticles as Potential Fiducial Markers for Image-Guided Radiation Therapy[J]. ACS Applied Bio Materials, 2022, 5(3): 1259-1266.
3. Liu H., Miyamoto N., Mai, NT., Shirato, H., Yonezawa, T. Green and Effective Synthesis of Gold Nanoparticles as Injectable Fiducial Marker for Real-Time Image Gated Proton Therapy[J]. Materials Advances, 2022, 3(13): 5430-5441.
4. Liu H., Miyamoto N., Mai, NT., Shirato, H., Yonezawa, T. Injectable Fiducial Marker for Image-Guided Radiation Therapy Based on Gold Nanoparticles and a Body Temperature-Activated Gel-Forming System. Minor revision.

List of Attended Conferences

Conference	Date	Title
The 72nd Divisional Meeting of Division of Colloid and Surface Chemistry	Sep. 21, 2021	Alginate-stabilized Gold Nanoparticles Prepared in Microwave-induced Plasma in Liquid Process with Long-term Storage Stability for Potential Biomedical Applications
The 5th A3 Foresight Symposium on Organic/Inorganic Nanohybrid Platforms for Precision Tumor Imaging and Therapy	Oct. 13-14, 2021	Body temperature-activated <i>in situ</i> - formation gel for the delivery and fixation of alginate-stabilized gold nanoparticles for proton beam therapy

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