### Soft Science

**Liquid Metals Enabled Advanced Cryobiology: Development and Perspectives**

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Abstract

Cryosurgery and cryopreservation, as two important categories in cryobiology, have been impeded by the poor thermal conductivity of biological tissues or specimens. To improve this, diverse adjuvants, e.g., carbon-based materials, metallic nanoparticles, metallic oxide nanoparticles, etc., have been exploited to improve the heat transfer in heat-targeted region to increase the tumor elimination efficiency as well as the post-thaw viability of cryopreserved specimens. Nevertheless, these materials suffer poor thermal conductivities, controversial biosafety problems and high expense. Gallium and its alloys, as a class of room-temperature liquid metals (LMs), have been widely studied in the past decade for their low melting point, minor toxicity, outstanding transformability and conductivity. Integrated with these superior properties, they have
been widely applied in multiple fields, such as thermal management, flexible
electronics and soft robotics. Recently, our laboratory has been devoted to fusing LMs
with cryobiology and made a series of progresses. In this article, we will first briefly
introduce preparation pathways to LM-based functional nanomaterials and composites.
Then, how these materials realize improvement in biological heat transfer will be
presented, followed by the discussion about the biosafety of these materials, which is
an essential concern for the cryobiological field. Recent studies employing LMs in
advanced cryosurgery and cryopreservation will also be highlighted. The present
challenges and prospects of LMs towards further development in cryobiology will be
put forward to point out the possible research direction.

**Keywords:** Liquid metal, cryobiology, cryosurgery, cryopreservation, nanomaterials,
biomaterials

**INTRODUCTION**

The field of cryobiology have made significant progress and been drawing increasing
attention in the past decades since technologies derived from this category are closely
related to welfare of human kind, for instance, organ transplantation, supplementary
reproduction, scientific research, tumor therapy, etc. On the road of development of this
discipline, huge challenges had been puzzling scientists for a long time, however, in
recent years, the roaring development of materials science has enabled many
exhilarating progresses.

Cryosurgery is the technique to employ freezing to eliminate undesired tissues. The
intent of cryosurgery is to import cold energy to free target tissues (e.g., adipose, naevus,
tumors, etc.) while avert minor cryogenic injuries to normal tissues. Especially for
tumor therapies, due to its minor pain, less side effects to patients and less invasion,
cryosurgery has been promoted to replace a wide range of traditional treatments\(^1\).
However, due to the intrinsic complex constructions and anisotropic thermal properties
of living tissues, the frozen region is always unable to be precisely regulated and always
unable to conform with the erose tumor tissues\(^2\). In some cases, freezing region did
not fully cover the tumor demarcation or the low freezing efficiency failed to thoroughly kill the tumor cells. These technical faults caused recurrence occasionally afterward[1], while excessive freezing would ineluctably hurt surrounding healthy tissues and cause excess pain and side effects to patients. These defects were usually attributed to the ununiform and inefficient heat transfer inside biological tissues. In order to overcome this, adjuvants were employed to enhance the freezing efficacy and regulate the freezing zone to ensure the elimination of tumors and protect healthy tissues as much as possible[3,4]. At the same time, these adjuvants could also: 1) Carry and deliver drug molecules to target tissues as assisted therapy. 2) Enable in vivo imaging for tracing and monitoring the therapy[5]. Currently, the well-studied adjuvants mainly include natural molecular adjuvants (such as antifreeze proteins[6,7], tumor necrosis factor alpha[8], glycine[9], and so on) and nanoparticles, for instance, MgO[10,11], Fe₃O₄[4,12], Al[13] and Au[14]. Among these, nanoparticles are capable of enhancing heat transfer, homogenizing temperature distribution, and more importantly, regulating ice nucleation. However, rigid nanoparticles could poorly match soft biological tissues and are reckoned to possibly cause abnormal cellular proliferation or cytokines secretion[15]. Thus, a soft and conformal material that can have a milder interaction with tissues and thermal enhancement is desired.

Cryopreservation is an opposite destination to cryosurgery, instead of utilizing cryogenic media to cause damage, cryopreservation intends to converse biological structures, and is regarded as the most promising approach to eternal preservation of biological resources since low temperature causes metabolic inverting. However, this technique is also confronted with huge challenge to preserve vulnerable cells or tissues and large-volume organs or even living organisms[16]. The conventional slow-freezing method causes inevitable ice formation in biospecimens and extended exposure to toxic cryoprotectants (CPAs), which would arouse abnormality to cell phenotypes, attachment, gene expression and differentiations[17]. Thus, slow-freezing method could hardly be applied to large-volume tissues and organs[16]. The emergence of the vitrification technique seems to bring a silver lining. Vitrification aims to deeply cool
down biospecimens without any bulky ice formation during both cooling and warming processes \(^{[16,18,19]}\). Therefore, vitrification is considered as a prospective strategy to tackle subsistent challenges and make a breakthrough in long-term preservation of complex tissues and large organs \(^{[16]}\). The vitrification can be described as a “racing competition” between the cryogenic thickening effect as well as the crystallization of water molecules. As long as the cooling rate is high enough to ensure the energy of water molecules inactive enough to fail organizing in order (i.e., formation of hydrogen bonds), successful vitrification is achieved and no considerable ice will form to damage cells. From this perspective, it is comprehensible that high-concentration CPAs are essential to increase the viscosity and suppress ice nucleation and growth. However, the resuscitation of vitrified biospecimens is rather challenging than cooling, because the ice nuclei forming during the cooling process tend to burst out growing during rewarming process. Cooperatively, the cooling and warming process both need to be rapid enough to ensure the phase change to overcome ice formation. Usually, for a certain CPA recipe, the critical warming rate (CWR) was several times higher than the critical cooling rate (CCR) \(^{[20]}\). The gold-standard water bath rewarming could hardly meet the criterion. Therefore, the idea of utilizing specific stimuli-responsive material-mediated heating effects to heat up cryopreserved biospecimens was proposed. J.C. Bischof and his colleagues developed and promoted nano-warming, which enabled ultra-fast rewarming of vitrified biosamples. They took advantage of the inductive heating effect of iron oxide nanoparticles (IONPs) to warm vitrified biosamples uniformly and rapidly and successfully kept their viability in both 1-mL and 50-mL systems \(^{[21]}\). In the same year, a laser-induced photothermal (PT) conversion of gold nanorods (GNR) was demonstrated to provide ultrahigh warming rate and facilitate the vitrification of zebrafish embryos \(^{[22]}\). In 2020, they studied the rapid rewarming of tissues based on the inductive heating of thin metal forms \(^{[20]}\). These were encouraging advances, they not only displayed ultra-rapid rewarming rate that exceeded CWR by orders of magnitude, but also showed the possibility of vitrification protocol applied to large-volume specimens. However, the mismatch between rigid metallic materials and soft biological tissues as well as cellular uptake phenomenon still remains, arousing
concerns about mechanical damage and metabolic safety of these materials. Moreover, preparing nanoparticles of noble metals is always complicated and costly. Because of the forecited reasons, there is an urgency to explore a soft, flexible, easy-to-mold and biocompatible material to supplement the vitrification technique.

Liquid metals (LMs) with low melting points have been drawing increasing interest in the past decades. Mercury is commonly used in routine life to be applied in thermometers, its alloys and compounds have also been developed as dental fillers, cosmetics additives and medicines\cite{23}. Alkalis alloys also have subzero melting points and have been applied as a heating source in hyperthermia therapy of carcinomas\cite{24,25}. However, these chemically unstable, hazardous substances require extra elaborative storage and operation, which restrict the application scope in biological field. Recently, several studies have spotlighted gallium (Ga)-based LMs with superior chemical stability, low vapor pressure, well biocompatibility\cite{26}. The authors’ laboratory has been focused on studying fundamental behavior, characteristics and applications in multiple fields. Based on previous research basis on cryobiology, our laboratory has been exploring the possibility of employing LMs into cryobiology to realize better outcomes. Thus, this review will give attention to only Ga-based LMs.

LMs features inherently high thermal conductivity and flexibility simultaneously. They have been widely exploited as effective thermal interface materials (TIMs) to enhance interfacial heat transfer\cite{27-29}. Through simple manipulations under room temperature and ambient pressure, LMs can be prepared into multiform combinational materials, including micro-/nano-scale LM particles with various morphologies\cite{30}. The transformability of liquid metal micro-/nano- particles (LMMPs/LMNPs) enables them to be applied in multiple fields, including printed electronics, drug delivery, biological sensing, etc. Moreover, resembling noble metal nanoparticles, LMNPs are also capable of displaying localized surface plasmon resonance (LSPR)\cite{31}, endowing them with high-efficiency PT conversion capability. Verified by a few works, LMNPs showed considerable PT effect\cite{32} and hence enabled efficient elimination of cancer cells and
inhibited the regrowth of tumors\textsuperscript{[33-37]}. Except for transforming into nanoparticles independently, LMs could also accommodate a wide range of micro-/nano- particles to form LM composites with modification of specific characteristics\textsuperscript{[38]}. In this perspective, the basic properties of LMs will be briefly introduced, interfacial issues are involved in almost all the manipulation of LMs and they will be emphatically discussed. Then, based on these impressive properties and phenomena of LMs, their recent advanced applications in cryobiology will be presented; lastly, from the perspective of future development of LMs in cryobiology, possible future application scope will be proposed.
Figure 1. Summarized introduction regarding cryosurgery and cryopreservation. The challenge and the current solutions are presented. The basic properties of emerging LMs are listed, which is thought to be a new solution for the development of cryobiology.

LM-BASED FUNCTIONAL MATERIALS
Preparation of micro/nano LM

The liquid nature of LMs enables them to be easily prepared into various modalities (Figure 1A). Compared to rigid metallic nanoparticles, which are practically synthesized by reductive method, LMs could be scaled-down through a facile top-down strategy. To obtain LMMPs or LMNPs, probe-sonication method is always applied. Generally, bulk LM is placed in a basic solution (e.g., deionized water, ethyl alcohol), then the ultrasonic probe induces cavitation to smash LM into smaller particles with oxide skin covered. During the sonication process, small particles still have the possibility to collide with each other and coalesce together to form back larger particles. Several factors, such as activation power, sonication time and temperature can influence the sonication efficiency and the size of particles. At the beginning of sonication, the mean diameter decreased as the time extended, some large bulk remained unbroken in this state. With sonication kept acting, the mean diameter became smaller till a minimum value. The mean size of LMNPs was highly correlated to LM type, solvent properties, temperature, mass ratio, etc. The sonication power has no impact on the eventual size of LMNPs, but only affects the time spent to reach the eventual state. High temperature tended to result in a larger particle size, which was probably attributed to the decrease of the cavitation effect, reducing the break-up ability of sonication.

In practice, for the purpose of acquiring relatively uniform and small LMNPs, static or centrifugal precipitation of large particles is always essential (Figure 2A).

LMs incorporated with other micro/nano particles

Despite the intrinsically superior merits of LMs, researchers still sought to further improve their performance in multiple aspects. Fortunately, the liquid nature makes LMs a universal platform to combine with diverse materials through simple mechanical agitation method. Chang et al. revealed the mechanism underlying this process. During agitating the mixture of LM and nanoparticles, air-induced gallium oxide skin was continuously cracked into pieces and new oxide would occur. Nanoparticles could hardly enter the bulk of LM because of the intrinsic high surface tension. However, they were gradually wrapped by sticky oxide debris during the stirring process thus LM
could easily internalize the nanoparticles\textsuperscript{[43]}. In this way, LM could incorporate various functional nanoparticles (Figure 2B), as summarized in Table 1.

Ga-based LMs could also incorporate with metallic nanoparticles through intermetallic wetting, which is induced by the formation of strong metallic bonds. Tang \textit{et al.} disclosed the intermetallic wetting-induced LM phagocytosis of copper nanoparticles\textsuperscript{[44]}. On the basis of experimental phenomena of biomimetic phagocytosis behavior of LM droplets as well as theoretical calculation of surface free energy, they concluded that: 1) The autonomous internalization behavior was a complete wetting phenomenon. 2) Irreversible intermetallic wetting provided sufficient force to overcome the energy barrier required for the transition from non-wetting to wetting. In another work by Tang \textit{et al.}, a typical intermetallic phase, CuGa\textsubscript{2} was observed and characterized to exist stably in GaIn-Cu composite\textsuperscript{[45]}. Doping with copper microparticles led to an impressive alteration in mechanical properties, thermal and electrical conductivities and melting/solidification points. In this manner, the characters of LM composites could be regulated more precisely, compared to the agitation approach. Adopting this technique, GaIn-Cu, with improved thermal and electrical conductivity has been applied to flexible electronics\textsuperscript{[46]} and enhanced cryoablation\textsuperscript{[47]}.

Following the external voltage-facilitated Cu particles doping approach proposed by Tang \textit{et al.}, Hou \textit{et al.} prepared GaIn-Cu composite to enhance the cryoablation efficacy\textsuperscript{[47]}. This principle also provided inspiration for Park \textit{et al.} to introduce LM-lyophobic carbon nanotubes (CNTs) to be finely incorporated with EGaIn, where Pt was selected as an intermediary agent\textsuperscript{[48]}.

\begin{table}[h]
\centering
\caption{Examples of LM incorporated with micro-/nano- particles.}
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Liquid Metal Composition} & \textbf{Doped particles} & \textbf{Approach (reagent)} & \textbf{Doping content (wt\%)} & \textbf{Improved properties} & \textbf{Reference} \\
\hline
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\end{table}
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<tr>
<th>LMMP/LMNPs</th>
<th>Process/Reagent</th>
<th>Sedimentation Method</th>
<th>Adhesion Efficiency</th>
<th>Transformability of LMMPs/LMNPs</th>
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<tr>
<td>GaIn&lt;sub&gt;24.5&lt;/sub&gt; Ni MPs</td>
<td>Mechanical stirring</td>
<td>3 - 9%</td>
<td>Adhesion [43]</td>
<td></td>
</tr>
<tr>
<td>GaIn&lt;sub&gt;24.5&lt;/sub&gt; Cu MPs</td>
<td>Voltage facilitation (NaOH&lt;sub&gt;aq&lt;/sub&gt;)</td>
<td>5 – 17%</td>
<td>Adhesion [45]</td>
<td></td>
</tr>
<tr>
<td>GaIn&lt;sub&gt;24.5&lt;/sub&gt; Cu NPs</td>
<td>Mechanical stirring</td>
<td>5 – 20%</td>
<td>Adhesion [46]</td>
<td></td>
</tr>
<tr>
<td>GaIn&lt;sub&gt;24.5&lt;/sub&gt; Cu MPs</td>
<td>Voltage facilitation (NaOH&lt;sub&gt;aq&lt;/sub&gt;)</td>
<td>29%</td>
<td>Adhesion [47]</td>
<td></td>
</tr>
<tr>
<td>GaIn&lt;sub&gt;24.5&lt;/sub&gt; Pt-CNTs</td>
<td>Mechanical stirring; NMP</td>
<td>3 – 15%</td>
<td>Adhesion [48]</td>
<td></td>
</tr>
<tr>
<td>GaIn&lt;sub&gt;24.5&lt;/sub&gt; Quartz MPs</td>
<td>Mechanical stirring or ball milling</td>
<td>UTD</td>
<td>Printability and recoverability [49]</td>
<td></td>
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<tr>
<td>GaIn&lt;sub&gt;24.5&lt;/sub&gt; Mg MPs</td>
<td>Mechanical stirring</td>
<td>0.5 – 3%</td>
<td>PT conversion efficiency and shapeability [50]</td>
<td></td>
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<tr>
<td>GaIn&lt;sub&gt;24.5&lt;/sub&gt; Fe NPs</td>
<td>HCl&lt;sub&gt;aq&lt;/sub&gt;</td>
<td>UTD</td>
<td>Magnetization [51]</td>
<td></td>
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UTD: Unable to determine; NMP: N-Methyl-2-pyrrolidone

**Transformability of LMMPs/LMNPs**

Due to large surface tension, the oxide-covered LMMPs/LMNPs are typically spherical. However, under specific conditions, LM particles could be shifted into diverse shapes. These anisotropic nanoparticles own specific optical, electrical and thermal
The shape-transformation of liquid metal particles could be realized by multiple means. Lin et al. sonicated EGaIn in deionized water containing positively charged surfactants and obtained liquid metal nanospheres. Then they heat the sample at 70 °C for 30 minutes and the nanospheres transformed into nanorods. They further manifested that such morphology shift was attributed to production of gallium hydroxide (GaOOH). Gan et al. prepared polydopamine-coated LMNPs and took advantage of the PT conversion capability of polydopamine to heat inner LM, triggering shape morphing of LMNPs from spheres to ellipsoids. Li et al. executed sonication with relatively high power (800 W) and extended duration (120 min) to maximize oxidization and obtained LM nanorods. Sun et al. sonicated LM in aqueous solution containing cetrimonium bromide, a positively charged surfactant, to obtain gallium and EGaIn nanorods. Besides sonication methods, due to the liquid nature of gallium, Wang et al. adopted a pressure-derived filtering approach to fabricating homogeneous gallium nanorods at 35 °C. Different morphology of LMNPs will bring about different thermal properties (e.g. thermal conductivity due to the composition change, specific absorption rate, photothermal efficiency, etc.) and biodegradability.

Sun et al. reported an intriguing transformation behavior of LMMPs under a space-restricted two-phase condition. When cooling the LMMPs in solution with a higher melting point, the basal solution would first solidify and trapped LMMPs. Then, with the temperature continuously dropping, due to the abnormal volume expansion phenomenon, LMMPs would expand and explode to form spikes to pierce surrounding ices. This phenomenon offered a distinct inspiration to exert excess mechanical damage to tumor tissues in cryosurgery. (Figure 2C)
Figure 2. (A) Preparation of micro/nano LM through sonication. a. Core shell structure of LM particles. b. The influential factors of the LM particles size after sonication. (B) LMs incorporated with metal particles and the main mechanisms. Mechanism 1: Metal particles internalization through sticky LM oxide. Mechanism 2: Metal particles internalization through the formation of metallic bonds. (C) Transformability of LMMPs/LMNPs. a. Influential factors on the
structure of LM particles. b. Cold induced transformation and the change of the interfaces between LMs and water.

**THERMAL PROPERTIES OF LM**

With the development of cryobiological techniques, precise and quick temperature control is essential to acquire optimistic outcomes. However, it is hindered by intrinsic low thermal conductivity of biomaterials themselves\textsuperscript{[56]}. Inferior heat transfer would result in failures in both cryoablation and cryopreservation, *e.g.*, insufficient elimination of cancer cells, damage to healthy tissues, ice recrystallization during thawing the vitrified biosamples, *etc*. To tackle this challenge, scientists added adjuvants to improve the heat transfer and homogenize heat distribution. More ideally, nanomaterials with self-heating effects (*e.g.*, GNRs, Fe\textsubscript{3}O\textsubscript{4} nanoparticles) have significantly increased the tumor ablation efficiency and preserved post-thaw cellular viability. Liquid metals, with high thermal conductivity and superior transformability, are prospective alternatives to conventional rigid adjuvants. In this section, two modes to enhance heat transfer of biomaterials and tremendous stimuli-responsive self-heating effect of LMs will be presented.

<table>
<thead>
<tr>
<th>Material</th>
<th>Melting Points (°C)</th>
<th>Thermal Conductivity (W·m\textsuperscript{-1}·K\textsuperscript{-1})</th>
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<tr>
<td>Water</td>
<td>0.0</td>
<td>0.599</td>
</tr>
<tr>
<td>Ga</td>
<td>29.8</td>
<td>29.4*</td>
</tr>
<tr>
<td>GaIn\textsubscript{20}</td>
<td>16.0</td>
<td>26.58</td>
</tr>
<tr>
<td>GaIn\textsubscript{24.5}</td>
<td>15.5</td>
<td>26.58</td>
</tr>
<tr>
<td>Ga\textsubscript{67}In\textsubscript{20}Sn\textsubscript{12.5}</td>
<td>10.5</td>
<td>39</td>
</tr>
<tr>
<td>Hg</td>
<td>-38.9</td>
<td>8.34</td>
</tr>
<tr>
<td>Au</td>
<td>1064.2</td>
<td>317</td>
</tr>
<tr>
<td>Fe</td>
<td>1538.0</td>
<td>80</td>
</tr>
<tr>
<td>Cu</td>
<td>1083.4</td>
<td>401</td>
</tr>
</tbody>
</table>
Skin - 0.29※
Muscle - 0.60※
Blood - 0.48※

*: Measured at 50 °C. ※: Data from reference No.[56]

Enhanced heat transfer

The inferior thermal conductivity of biomaterials is an inevitable barrier for regulation of their temperature with rapid response, wide range and high precision. In the past two decades, our laboratory has been deeply exploring LMs as coolant[57-60] or thermal interface materials[27-29,61], which has confirmed LMs’ ability to enhance heat transfer and diminish contact thermal resistance (Figure 3A). For biomedical application, Wang et al. prepared EGaIn-based composite pastes to actualize thermal therapy to subcutaneous tumors[50,62]. The modified LMs were tightly adhesive to skins and increase the heat penetration into tumor tissues, according to the simulation results. Hou et al. testified the probe cryoablation and manifested the increased “cold energy” transfer enabled by conformally coated LM paste in both numerical simulation and experiments[47].

Besides bulk LM enabled heat transfer improvement, the idea of “nano-cryosurgery” has been early proposed, which injects solutions containing nanoparticles (i.e., nanofluids) into tumor site to increase heat transfer, aggravate freezing damage and regulate ice ball formation. For a solution containing nanoparticles, its thermal conductivity can be estimated by Maxwell-Garnett’s model[63]:

\[
\frac{k_{\text{eff}}}{k_{\text{base}}} = \frac{(1 - \varnothing)(k_{\text{np}} + 2k_{\text{base}}) + 3\varnothing k_{\text{np}}}{(1 - \varnothing)(k_{\text{np}} + 2k_{\text{base}}) + 3\varnothing k_{\text{base}}} \tag{1}
\]

where, \(k_{\text{eff}}, k_{\text{base}}\) and \(k_{\text{np}}\) are the thermal conductivity of nanoparticle-dispersed solution, basal solution and dispersed nanoparticles, respectively, \(\varnothing\) is the volume fraction of nanoparticles. According to this model, we can speculate that with higher
concentration and higher intrinsic thermal conductivity of nanoparticles loaded, the thermal conductivity was significantly increased. Di et al. implemented nanoparticles-mediated cryosurgery and figured out that MgO nanoparticles could significantly increase the thermal conductivity of tissue and promote the ice ball formation. The histological characteristic of frozen area also indicated that MgO enhanced cryosurgery to biological tissues. Hence, surgeons can regulate the ice formation in spatial and time domain by injecting nanoparticles of specific concentration and in certain regions[1].

Fan et al. dispersed LMNPs into methyl silicone oil, acquiring improved thermal conductivity and more efficiently heat dissipating effect.

**Stimuli-responsive heating effect of LMs**

For vitrified cryopreservation, sufficiently quick and uniform heating of biospecimens is key to the success of vitrification. In addition, high concentration of CPA with low critical cooling/warming rate is required. To meet the criteria, containers with tiny volume and large contact area were designed to reach high cooling/warming rate. However, it would largely restrict the practical application of vitrification technique. To tackle this challenge, scientists added adjuvants with self-heating capability in CPA recipes to accelerate thawing the biospecimens. Several nanomaterials have been manifested as PT (e.g., GNRs[22], MoS$_2$ nanosheets[64], MXene nanosheets[65], etc.) or magneto-inductive agents (e.g., iron oxides[21,66,67], metallic foams, foils and meshes[68]) to successfully thaw vitrified biospecimens. Compared to conventional convective rewarming method, this technique features many superiorities: i) non-contact, ii) tunable and extremely high heating rates, iii) uniform temperature distribution, iv) biospecimen scaling-up and v) potential in reducing the dose of CPA, etc. Studies in recent years have demonstrated LMs with capability of self-heating in both modes, which will be presented in this section, as shown in Figure 3B.

**Photothermal effect**

The PT effect of plasmonic nanoparticles is attributed to localized surface plasmon resonance. Briefly speaking, the alternating electrical field in the incident light triggered
dipolar oscillation of free electrons, thus arousing resonance of internal lattice and generating heat. Chechetka et al. revealed the profound PT effect of LMNPs and verified its efficient damage to cancer cells\cite{42}. They claimed the PT conversion efficiency as high as 52%, which is much higher than commercially used AuNR1 (17%) (Figure 3C). Sun et al. prepared variform LMNPs and characterized their morphology-dependent PT conversion efficiency\cite{33}. The stability of PT agents is always of great concern, especially considering the fact that temperature rise causes oxidization and morphing of LMNPs, as discussed earlier in this paper, which would cause exacerbation in efficiency. Wrapped with non-PT coatings, LMNPs were able to resist shape morphing and realize stable repeated self-heating. Hu et al. manifested that LMNPs coated with mesoporous silica displayed enhanced immobilization and sustained PT effect\cite{69}. Besides direct hyperthermal damage to cancer cells, the PT effect could also cooperate with chemotherapy or embolization to realize synergistic treatment (Figure 3D). Grafted with functional ligands, LMNPs could carry anti-tumor drugs and release them when triggered self-heating under irradiation, which further improve the tumor elimination efficiency\cite{42,69,70}. Wang et al. integrated PT treatment, chemotherapy and embolization in one combinational material\cite{51}. They successfully encapsulated EGaIn-Fe nanoparticles and doxorubicin hydrochloride into alginate hydrogels as microspheres. This hybrid agent could be immobilized at main artery of tumor, perform photothermal effect under irradiation and thus trigger drug release to target tumor tissues.

Inductive heating

In recent years, magnetic inductive heating has been studied in tumor therapy and vitrified cryopreservation. Compared to PT technique, of which the actuation range is restricted by irradiation area, magnetic inductive heating holds two major superiorities: i) larger actuation scale, ii) better flexibility of manipulation. Fe$_3$O$_4$ is the most applied magnetic agent for inductive heating. Manuchehrabadi et al. applied Fe$_3$O$_4$ nanoparticles and manifested the capability of magneto-inductive heating to rapidly and uniformly thaw vitrified arteries up to 50 mL\cite{21}. Zhan et al. applied this technique with
carboxylic acid-modified Fe$_3$O$_4$ to resuscitate cryopreserved whole rat kidneys and achieved integral structures. As metallic materials, LMs are also appropriate for this technique. Under alternating magnetic field, eddy current will be produced due to Faraday’s law of induction and thus generating heat inside metal. Wang et al. demonstrated a more considerable heat generation of LM than Fe$_3$O$_4$ in alternating magnetic field$^{[71]}$ (Figure 3C). Then, by modifying LM with PEG and DOX, they realized thermo-chemo hybrid therapy. In another work, Wang et al. prepared oxidized EGaIn to conformally coat on mice skin and generate considerable heat under alternating magnetic field to actualize thermal therapy to subcutaneous tumors$^{[62]}$. With such highly efficient inductive heating effect, LMs hold great potential in vitreous cryopreservation.

Figure 3. (A) Remarkable interaction between LMs and biomaterials. a. soft interaction between LMs and biomaterials without any stiff damage. b. Conformal adhesion between LMs and biomaterials. (B) The ability for LMs to enhance the heat transfer in different modality under various conditions. (C) Superior stimuli-responsive properties of LMs over commercial nano-materials. (D) Synergistic treatment of combining thermal property of LMs with chemotherapy and embolization.
BIOSAFETY

For biological applications, the safety of materials always stands as the top priority. Unlike mercury, which easily vaporizes in the atmosphere and causes inhalation risk, Ga-based room-temperature LMs have extremely low saturated vapor pressure, thus requiring no special packaging for long-term storage. Despite the natural stability, the toxicity of LMs in biological environments still attracts great attention. Both in vitro and in vivo toxicity of LMs, whether in bulk formation or particles, will be discussed in this section.

Ex vivo cytotoxicity

For Ga-based LMs, the major factor that causes cellular toxicity is ionic release. Kim et al. systematically evaluated the ionic toxicity of eutectic Gallium–Indium (EGaIn) in the aqueous environment\cite{72}. For bulk EGaIn, Ga$^{3+}$ concentration increased with soaking time until saturated, while In$^{3+}$ stayed at a negligible level. The cytotoxicity was evaluated by pre-soaking LMs within the growth media for 24 hours, followed by co-culturing cells with these media for different days and viability characterizations. Their results showed that bulk EGaIn had little impact on the cellular viability and proliferation. However, when LMs were sonicated to micro-/nano- particles, the concentration of In$^{3+}$ was elevated over 1,000 times within 20-minute sonication (Figure 4A). This was probably ascribed to the increased surface area-to-volume ratio, which resulted in increased interfacial interaction between LMs and solutions. This process also allowed more In to appear at the surface of LM particles and to interact with the solution. In vitro assays indicated significant damage to cells caused by releases from LM nanoparticles (with a mean size of less than 500 nm) within only 1-day co-culture (Figure 4B). Given the fact that ultrasonication caused significant change in In$^{3+}$ rather than Ga$^{3+}$ and it led to decline in cellular metabolic activity, it could be speculated that In$^{3+}$ gave rise to more critical cytotoxicity than Ga$^{3+}$.

In recent years, there have been much research that adopted LMs in biomedical applications and studied their biosafety in different hierarchies. At the cellular level,
their results were generally in accordance with the above discussion. Large-scale LMs tend to show no significant damage to various cell lines\cite{62,73,74}. Whereas LM nanoparticles showed more or less damage to cells, e.g., nanospheres prepared by Hou et al. and nanorices prepared by Yan et al. both exhibited inhibition to cellular metabolic capability\cite{75,76}. However, it is noteworthy that Hou et al. only took a small amount of suspension after sonication to operate lyophilization and later re-dispersed LMNPs in cell culture media, which resulted in the dilution effect to relieve their in vitro toxicities\cite{75}. As conjectured by Kim et al., the sonication process generated extremely localized heat and pressure as well as induced chemical reactions, which might encourage ion release\cite{72}. Then we can regard the eventual ion number of LM nanoparticles suspension as two parts: i) the ion number right at the timepoint that sonication terminated (n_{son}) and ii) the number of ions released during the static standing of the post-sonication suspension (n_{sta}). According to the result of Kim et al., we can speculate that n_{son} >> n_{sta}, indicating that dilution of primary dissolvent could probably remit the ionic toxicity. To further tackle the ion-release problem, biocompatible encapsulation was applied to restrain the release of ions, e.g., Wang et al. prepared calcium alginate hydrogel packaged magnetic LM nanoparticles and verified their negligible toxicity to different cell lines with the concentration from 2.5 to 200 mg/mL\cite{51} (Figure 4C).

\textit{In vivo biocompatibility}

Although the cellular toxicity of bulk LMs (or encapsulated LM particles) was evidently proved to be negligible, the in vivo biosafety was still of great concern. Cogitation includes inflammatory reaction, mechanical damages, the metastasis of materials and concentration effect, organ toxicity and hematic toxicity, etc.

A few works also systematically evaluated the influence of in vivo introduction of LMs. Yang et al. operated a subcutaneous injection of 20 μL EGaIn and made a biopsy of vicinal tissues\cite{77}. H&E staining images indicated neither obvious morphological alteration of skin or muscles near the injection site, inflammation, nor muscular
degeneration was found (Figure 4F). In addition, the injected LM showed remarkable stability and barely metastasized to the main organs. Except for subcutaneous injection, Wang et al. also injected a larger amount (100 μL) of calcium alginate encapsulated EGaIn through the tail vein (TVI) of Balb/c mice and measured the Ga and In distribution after days of injection[51]. They found that TVI not only resulted in a higher concentration of ions in main organs but also caused delayed concentration peak time in almost all the organs after injection. Nonetheless, the levels of both Ga and In ions were still within a tolerable range and could be deemed as non-toxic to living bodies. As metabolically active organs, liver and kidney are easily damaged by exogenous toxins. To evaluate the hepatic toxicity, two representative indicators, AST and ALT levels are especially detected. There was no evidential increase in both enzyme levels (Figure 4D), reflecting no hepatic dysfunction[62,77]. Wang et al. also demonstrated that the concentrations of urea and creatine, which reflect renal function, stayed in a safe range (Figure 4E). As an overall criterion for in vivo safety, the body weight of animals was continuously monitored after LM injection, turning out there was no significant influence on the body weight[35,51,73] (Figure 4G).
ADVANCED APPLICATIONS OF LM IN CRYOBIOLOGY

Integrated with all the properties discussed above, LMs have the potential in application towards two opposite directions. Herein, we will demonstrate that LMs can either serve as ice inhibitors to protect biosamples during cryopreservation or exert enhanced damage to tumor cells in thermotherapy, determined by specific manipulation. Our laboratory has been striving to push forward the application of LMs in cryobiological applications and made some significant progress in recent years, which will be presented in this section.

LM-mediated vitrification of cell suspensions

Aiming of no ice formation through the whole procedure, vitrification has been more widely investigated and holds the prospect of increasing viability of cells and scaling up the dimension of cryopreserved specimens\textsuperscript{18,20,21,64,66}. On account of the acknowledged fact that the rewarming process of this technique is confronted with more austere challenges than the cooling process, high rewarming rates are crucial to successful vitrification. Inspired by the significant PT conversion effect of LMNPs, Hou
et al. applied them as nanoscale heat sources that realized uniform and rapid rewarming of vitrified cell suspensions\textsuperscript{[75]}. They utilized ultrasonication to prepare LMNPs, followed by the centrifugal screening of particles and lyophilization of supernatant, which ensured a uniform size distribution of LMNPs. The in vitro biocompatibility test drew the conclusion in accordance with the works presented above. The calculated PT conversion efficiency was 52%, which was higher than long-studied gold nanomaterials and in accordance with the previously reported value\textsuperscript{[42]}. This consistency probably also suggested that surface modification might have little influence on the PT efficiency of LMNPs. Benefiting from the “nanowarming” technique of LMNPs, the cryoprotectant (CPA) recipe they applied was free of DMSO, which is widely considered to be cellular toxic\textsuperscript{[78]}; at the same time, the total CPA concentration was far lower than commercial recipes\textsuperscript{[20]}. With a minimum dose of LMNPs (0.1 mg/mL), the viability of rewarmed cells exceeded 70%. Even in the experiment groups without laser irradiation, the cell viability was still uplifted with a higher dose of LMNPs, probably ascribed to heat transfer enhancement of the suspension. The resuscitated stem cells were evaluated in multiple dimensions, including attachment efficiency, proliferation, expression of critical antigens and genes, as well as multi-directional differentiation ability. For proof of the potential of this technique in large-scale specimen cryopreservation, they further vitrified and rewarmed murine tails, resulting in better protection efficacy from the view of histological morphology analysis.

This work primarily exploited LMNPs as functional materials in cryopreservation protocol, which mainly made differences from three aspects: i) No DMSO was added to the CPA recipe. ii) Dispersing uniformly in CPA to increase the thermal conductivity of suspension. iii) Serving as PT sensitizers to rapidly rewarm the vitrified specimens to avoid recrystallization. Combining their thermal properties, modifiability, softness and biocompatibility, LMs hold great prospects in the field of cryopreservation.

**LM-enhanced tumor thermotherapy**

LM-mediated ice-fire ablation
Combining high thermal conductivity, PT effect and conformability, Hou et al. proposed an LM-based hybrid platform to improve the efficacy of tumor therapy\cite{47}, which comprised two major parts: i) They prepared pasty LM-Cu composite to conformally coat onto skin to enhance the heat transfer. As shown in Figure 5B(i), the simulation result indicated that LM paste coating could result in deeper penetration of cold energy into tumor. ii) They injected LMNPs into tumor and radiated the tumor with near-infrared ray (NIR) to implement photothermal therapy (PTT) (Figure 5B(ii)). The near-infrared thermograph demonstrated phenomenal PTT effect aroused by LMNPs. Furthermore, to further exploit the therapeutic effect of LM platform, Hou et al. combined these two modes successively. The tumor cells underwent drastic temperature change from extreme hypothermia to hyperthermia, which would also cause intensive thermal stress to ultimately kill tumor cells. The experimental result manifested that LM platform mediated cryoablation combined PTT realized better tumor elimination efficiency than each therapy alone. Besides, only in combined therapy group, the tumor did not undergo recurrence.

**LM deformation enhanced destruction to tumors**

Sun et al. discovered an intriguing deformation behavior of LMMPs in a dual-liquid phase system. Though the melting point of gallium is 29.8 °C, however, due to the size-dependent supercooling effect, Sun et al. characterized the phase transition temperature as between -20 °C to -60 °C, which is much lower than the chitosan solution\cite{73}. Therefore, when the temperature decreased, the chitosan solution first froze and LMMPs were trapped inside ice. When LMMPs underwent phase change, they would expand and form spiny deformations to cause mechanical damage to surrounding ice within only 1 ms\cite{73}, as shown in Figure 5C(i). The deformation ratio of LMMPs is determined by the property of surrounding solution (Figure 5C(ii))\cite{55}. In this way, by simply employing LMMPs as cryosurgery agents, an enhanced cryoablation efficiency would be realized. Sun et al. exerted cryoablation to C8161 tumor in nude mice model and, as shown in Figure 5C(iii), they demonstrated an improved tumor elimination efficiency and higher survival rate in LMMPs injection group.
In order to increase the targeting ability and increase the destructive efficacy, Wang et al. assembled cell membrane wrapped Ga particles (Ga/MPs) with the ability of being delivered into cancer cells to exerted cactus-like deformation inside endosomes under freezing to cause endosomal escape[79] (Figure 5D (i) and (ii)). They demonstrated prominent targeting capability of Ga/MPs through fluorescent staining tracing method. The fluorescence microscopic images show co-localization of endosomes and Ga/MPs. After freezing treatment, the co-localization area significantly decreased, indicating more endosomal escape caused by Ga/MPs. With injection of Ga/MPs, the cryosurgery efficiently inhibited tumor growth, compared with the outcome of group without cryosurgery. In addition, without membrane wrapping, the cryosurgery inhibited tumor growth with a slighter effect compared to Ga/MPs combined cryosurgery group. Such distinct comparison verified the enhanced anti-tumor effect deriving from Ga particles deformation. To further improve the therapy effect, a combined chemotherapy was made by loading antitumor drugs with Ga/MPs and resulted in better tumor elimination effect (Figure 5D(iii)).

These works indicated that LMs, with various forms, could be employed as synergistic platforms to exert multi-mode tumor therapy, especially to enhance cryoablation efficiency.
Figure 5. Advanced cryobiological applications of LM-based materials.

A. LMNPs-mediated ultrarapid rewarming of vitrified biospecimens. Reproduced with permission[75] Copyright 2019 Acta Materialia Inc. (i) Illustration of vitrification of cell suspensions loaded with LMNPs and NIR laser induced rewarming. (ii) Illustration of LMNPs inhibiting ice formation. (iii) Live/dead staining of resuscitated cells with/without LMNPs. B. LM-mediated combined cryoablation and PTT. Reproduced with permission[47] Copyright 2020, American Chemical Society. (i) Illustration of LM paste coating enhanced cryoablation. Inset is the simulated result of temperature distribution with (left) or without (right) LM paste. (ii) Illustration of LMNPs-mediated PTT. Inset is the in vivo infrared thermographic image of radiated tumor tissues bearing LMNPs (left) or not (right). (iii) Post-treatment cellular viability. (iv) Change of post-treatment tumor volumes. C. Freezing induced LMMPs deformation enhanced cryoablation. (i) Illustration of probe-cryoablation and LMMPs deformation enabled mechanical

PROSPECTS OF LM IN CRYOBIOLOGY

LM-mediated cryosurgery

As mentioned above, cryosurgery attracts increasing attention based on the characteristics of minimally invasive and lower side effects than traditional surgical treatment. Focusing on the key points during the operation, LMs have been applied to enhanced the biological heat transfer successfully, therefore achieve sufficient freezing to target tumor and show great potency in the animal model of melanoma. It has been reported that LMs can act as an extraordinary contrast agent for X-ray visualization, thus it is favorable to realize accurate cryosurgical guidance through solid tumor vascular network injection of LMs. Moreover, LMs are also prospective in synergistic immune therapy and direct tumor-killing by iron deprivation to boost the tumor killing efficiency when combining with cryosurgery, and all of which will be discussed in detail as follows.

LM-mediated imaging

Medical imaging refers to the technology and processing process of obtaining internal tissue images of the human body or a part of the human body in a non-invasive way for medical treatment or medical research. Since Roentgen discovered X-rays in 1895, imaging has been one of the most critical parts in clinical trials, which plays an
important role in diagnosing and preoperatively planning diseases that cannot be
directly observed. Tumors are highly heterogeneous with irregular shape and is
normally embedded deeply in tissues. Thus, these intrinsic properties of tumors pose a
serious challenge to surgical methods, including cryosurgery that rely on physical
elimination of tumor tissues. Meanwhile, inaccurate imaging guidance can easily lead
to uncontrolled cold release and cause damage to adjacent healthy tissues.

At present, most cryosurgeries are percutaneous ablation guided by CT or ultrasound,
which are widely used because of their simplicity and low cost\cite{80}. However, these two
imaging methods still have shortcomings in achieving high resolution and high contrast
intraoperative monitoring. With the increasing demand for precise and conformal
treatment of tumor cryoablation, developing intraoperative monitoring means of high
sensitivity and high resolution is of great significance to achieving accurate cryo-
biomedicine.

As a multifunctional metallic material, gallium also shows a wide range of application
prospects in biological imaging and can realize a multi-mode imaging ability, including
CT, magnetic resonance, and it even shows powerful capability in the emerging
photoacoustic imaging field\cite{42}. The characteristics of LMs contribute greatly to
imaging quality improvement. For example, LMs exhibit phenomenal X-ray absorption
and are greatly radiopaque due to the higher density (6.08 g/cm$^3$) than aqueous solution.
And gallium has been proven to be an excellent contrast agent for angiography, which
could allow even tiny capillaries to manifest in high resolution because of the fluidity
and compliance\cite{81}. In tumor treatment, LMs can be injected directly into tumor tissue,
while the high fluidity enables the LMs to disperse quickly at the target tissue, so as to
achieve complete and clear imaging of diseased tissues. In addition, LM can also be
injected through blood vessels around tumors. It is well known that tumor induces a
complex blood vessel network to scramble for nutrients and oxygen. Fan et al.\cite{82}
synthesized an injectable LM-calcium alginate hydrogel to realize local embolization,
which can not only cut off the energy supply for the tumor but also act as markers
around the tumor for cryosurgery killing boundary localization. Plenty of studies have
demonstrated the imaging potential of LMs. However, the realization of high-quality
intraoperative imaging guidance mediated by LM remains to be further developed.

Aiming at the problem of incomplete killing caused by unclear imaging of tumor tissue
during cryoablation so far, efficient intraoperative image mediated by LM is worthy of
expectation. It is worth expecting that LM-mediated intraoperative imaging will
provide potent medical guidance for the planning and ablation of cryosurgery to achieve
maximum tumor-killing efficiency and avoid tumor residual and recurrence (Figure 6A).

**LM-mediated synergistic immune therapy**

Adjuvant refers to a class of substances that can specifically bind to an antigen through
physical or chemical means to enhance the specific immunity of the antigen. In the past
few decades, a variety of adjuvants have been developed and applied in vaccine
manufacturing successfully. Thereinto, aluminum, as one of the most popular immune
adjuvants, has been widely used in vaccine manufacturing and other fields since it was
first discovered in 1926 by Glenny. Therefore, it is worth considering that gallium, as a
congener of aluminum, may also be an excellent immune adjuvant, as presented in
Figure 6B.

Immunotherapy is a potential research direction for cancer treatment in the future\(^8^3\),
with the aid of tumor vaccine to release the immunosuppressive state and stimulate the
patients’ own immune system so as to achieve the purpose of controlling and further
eliminating the tumor. A key role of immune adjuvants in tumor vaccines is to boost the
uptake and cross-presentation of antigen-presenting cells\(^8^4\). It is reported that LM
nanoparticle-based nano-vaccine can enhance antigen-presenting. Meanwhile, it can
produce moderate regional inflammation under infrared radiation, thus reinforcing the
immune system response as well as immune cell recruitment\(^8^5\). The vaccine shows an
evident suppression effect in mouse breast tumor models and is thought to be greatly
adaptive for multi-type cancer treatment. However, the underlying molecular
mechanism of this study still needs to be further explored.
Compared with traditional surgical resection and chemoradiotherapy, cryoablation can retain the antigenic activity of the tumor due to its cryogenic characteristics, which is crucial for relieving the immunosuppression of middle and advanced tumors and effectively initiating the anti-tumor immune response. The delightful result is on the strength of targeted killing to tumor cells as well as the recognition by the immune system after tumor cells rupture and release of antigens mediated by cryosurgery. Studies have also shown that cryoablation can induce the abscopal effect, which refers to the reduction or even disappearance of the tumor that has metastasized to other organs and tissue in the process of tumor treatment, and this effect has been proved by cryoablation for prostate tumors treatment\textsuperscript{[86]}.

Hence, it is worth expecting that the synergistic immune therapeutic effect of cryoablation and LM as well as its distinctive physical characteristics under cryogenic temperature will bring new prospect for cancer treatment.

**LM-mediated cancer therapy**

Aluminum, gallium and indium are three metallic elements in the periodic table with increasing atomic numbers in the same group IIIA. Thus, it is self-evident that they show certain similarities in chemical properties (Table 3). However, numerous *in vitro* and *in vivo* biological experiments have demonstrated that gallium and iron ions show more evident similarities in the physiological environment. And it is precisely due to the similar biochemical properties of the two ions that constitute the basis of a series of physiological effects of gallium so that it could be applied to many clinical occasions widely, including anticancer and antibacterial practices\textsuperscript{[87]}.

The high degree of chemical similarity between gallium and ferric ion is largely due to their proximity in ionic radius and bonding (Table 3). Therefore, in living organisms, Ga\textsuperscript{3+} can replace Fe\textsuperscript{3+} when it binds with certain proteins, such as transferrin\textsuperscript{[88]}, lactoferrin\textsuperscript{[89]} and ferritin\textsuperscript{[90]}, thus further blocking the implementation of corresponding functions. It is worth noticing that compared with Fe\textsuperscript{3+}, which can be efficiently converted into Fe\textsuperscript{2+} or vice versa, Ga\textsuperscript{3+} does not undergo the reduction reaction under
physiological conditions, which effectively prevents Ga$^{3+}$ from entering the protein bounded with Fe$^{2+}$ and blocks its function. Therefore, Ga$^{3+}$ does not enter the blood cells and bind with hemoglobin to affect the oxygen transport process$^{[87]}$.

In practical gallium compounds relating to cancer treatment, gallium ion is just based on the aforementioned principle, which competitively binds with proteins over ferric ion, block the physiological process of cancer cells in the presence of specific proteins so as to achieve its anticancer role (Figure 6C). Gallium shows a strong anti-mitotic ability of cancer cells. An important reason is that there are numerous transferrin receptors distributed on the surface of cancer cells$^{[91]}$, which can rapidly transport gallium into cells, resulting in the conformational change and inactivation of nucleotide reductase, which prevents DNA synthesis in cancer cells and stops cancer cell mitosis at S phase in cell cycle, ultimately prevents the massive proliferation of cancer cells and the iron-depriving inhibition effect has been observed in human acute lymphoblastic leukemia cells$^{[92]}$. Furthermore, iron-deprivation will induce apoptosis which enhances the cancer cell proliferation suppression$^{[93]}$. Although high concentrations of gallium ions can cause chromosomal abnormalities and nuclear damage, gallium shows low toxicity at therapeutic concentrations and can only be enriched in specific cells with high transferrin receptors.

The direct therapeutic effects in cancer treatment of gallium endow LM with great potential in cancer treatment when combined with cryosurgery. And it is promising that Ga$^{3+}$ will remedy incomplete tumor killing and prevent tumor recurrence after cryosurgery.

Table 3. Comparison among Ga$^{3+}$, Fe$^{3+}$, Al$^{3+}$ and In$^{3+}$

<table>
<thead>
<tr>
<th>Ionic radius (octahedral) (Å)</th>
<th>Ionic radius (tetrahedral) (Å)</th>
<th>Ionization potential (eV)</th>
<th>Electro-negativity (eV)</th>
<th>Metal-oxygen bond</th>
</tr>
</thead>
</table>

[Table content]
<table>
<thead>
<tr>
<th></th>
<th>dissociation energy (kJ mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ga(^{3+})</td>
<td>0.620 0.47 64 1.81 353.5</td>
</tr>
<tr>
<td>Fe(^{3+})</td>
<td>0.645 0.49 54.8 1.83 390.4</td>
</tr>
<tr>
<td>Al(^{3+})</td>
<td>0.535 0.39 119.99 1.61 511</td>
</tr>
<tr>
<td>In(^{3+})</td>
<td>0.800 0.62 54 1.78 320.1</td>
</tr>
</tbody>
</table>

This table is cited with permission from Bernstein\(^{[87]}\) published in Pharmacological Reviews.

**LM-mediated cryopreservation**

There are few studies concentrating on the application of LMs in cryopreservation, whereas the prospect to accomplish LM-mediated cryopreservation is intriguing. Based on one of the critical obstacles that tissues are unfavorable in thermal conductivity for achieving excellent cryopreservation, LMs are expected to accelerate the heat dissipation so as to rewarm biosamples uniformly and rapidly. The nanoscale LM has been presented that rapid warming rate was attained in vitrification through PT stimulation. However, the possible application of bulk LMs and the combination with various external fields is worth further exploration. It is also found that LM, specifically gallium, have the ability of anti-inflammation and immunomodulation, which endow it with great chance to alleviate freezing-induced inflammation and regulate transplant rejection.

**LM-mediated advanced cryopreservation**

The efficient preservation of biological samples is an important advance in human medical history. At present, there have been relatively sufficient studies and mature techniques for the preservation of cells and small-scale tissues. However, for large-scale
tissue and organs, there is still an unbridgeable gap regarding heat transfer between biological samples and temperature-controlling medium in the process of heating and cooling procedures. It is a practical method to enhance heat exchange through altering interface characteristics, which has been applied for heat dissipation of electronic devices. The modified interface between biospecimens and the outer solution will enhance the energy transport process directly. In addition, researchers also demonstrated that a variety of metal materials, such as metal foil, foam, and mesh, can achieve an impressive rapid warming rate of more than 1000 °C/min because of the remarkable induced eddy currents under the radiofrequency field that generate heating through concurrent resistive losses[^68].

For the purpose of conformally enhancing heat transfer as well as achieving a rapid warming rate, LM is superior to any other metals that had been tested in previous work, including copper and aluminum, because of the excellent intrinsic flexibility and shape adaptability. LMs can be quickly coated on the surface of biological samples before cryopreservation, and samples with irregular shapes can also be well coated without dead points, which avoids complex metal layer preparation and preservation defects caused by imperfect metal coverage (Figure 6D).

**LM-mediated anti-freezing induced inflammation**

Cryopreservation achieves long-term preservation by lowering the temperature to reduce cell metabolism. But low temperature is also a major cause of damage to biological samples such as organs when preserved by hypothermic storage or hypothermic machine perfusion. At the macro-level, possible organ damage includes the destruction of vascular networks and tissue structure, which is closely related to mechanical damage of ice crystals and thermal stress during cooling and rewarming[^94].

At the micro-level, the blocked blood flow for organs will trigger ischemic injury in the process of preservation. Long-term ischemia will lead to hypoxia, metabolic disorders, calcium overload and other a cascade of problems. In addition, mitochondria are the main target of hypothermia damage, which cause rapid adenosine triphosphate (ATP)
consumption and enzyme activity decrease, it is reported that around 95% ATP will be consumed in the first 4h when stored in 0~4 °C[95], which is harmful to the ATP-based ion exchange (e.g., Na⁺/K⁺-ATPase) and damage the cytoskeleton and membrane integrity, and then accumulated metabolic and ion imbalances will pose a serious threat to cell survival.[96] Low temperature also gives rise to the loss of cellular antioxidant capacity. Subsequently, the unbalanced antioxidant defense system induces oxidative stress in cells, producing large amounts of ROS, and ultimately leading to cell necrosis and apoptosis.[97] It is also found that gene transcription and protein synthesis are also affected by the expressions of apoptosis-sensitive genes such as Bcl-2/Bax and the activation of caspase series proteases with respect to cell apoptosis[98].

Meanwhile, the apoptotic and necrotic cells will induce the release of damage-associated molecular patterns (DAMPs)[99]. Due to the combined adverse condition above, organs are prone to suffer inflammation during and after preservation. Generally speaking, inflammation is an active protective response produced by the body in the face of pathogens, tissue damage and other adverse conditions. A range of immune cells, including T cells and macrophages, are involved in the inflammatory response and synthesis as well as release cytokines, which mediates a series of processes targeting immune stimulation. However, it is extremely unfavorable to undergo inflammation for a long time, which is easy to induce tissue dysfunction, cancer, necrosis and even death.

Gallium has shown great potential in immunomodulating, which gallium can suppress the reaction of the immune system without being cytotoxic. Bouissou and Maurel et al.[100] hypothesized that gallium can suppress the ability of immune cells, specifically T cells and macrophages. The subsequent experiment demonstrated that T cells and macrophages are two targets of gallium and gallium employs different impact mechanisms towards those two cell types. Gallium can inhibit T cell activation and proliferation at the early stage without toxicity, whereas gallium can temporarily restrain major histocompatibility complex (MHC) class II found in murine macrophages.[101] It is also noticed that the existence of gallium results in the reduction of cytokines. Inversely, gallium could upregulate anti-inflammatory cytokines, such as
IL-10, which is of great significance in inflammation resolution (Figure 6E)\textsuperscript{[102]}. 

**LM-mediated immunomodulation**

Organ transplantation is a historic progress in human medicine and health development, and it is the only effective method for the treatment of a great deal of end-stage diseases. Nowadays, the demand for organ transplantation is still unmet. Cryopreservation is acknowledged as a way to significantly extend the preservation time of isolated organs, which greatly alleviates the current contradiction between organ supply and demand. Beyond that, however, organ transplantation is still confronted with grim challenges. Many recipients are tortured by the serious immune rejection after receiving the transplanted organ, resulting in dysfunction of the graft, and acute immune reaction is even life-threatening. Therefore, immunosuppressants were introduced to alleviate the immune responses, which effectively improved the transplant prognosis\textsuperscript{[103]}. 

As mentioned above, gallium is thought to be an immunomodulating agent in relieving inflammatory responses, particularly the immune process mediated by T cells and macrophages. The unique property intrigue researchers whether gallium could act as a metal ion immunosuppressant to attenuate immunological rejection or not after transplantation. Orosz et al.\textsuperscript{[104]} applied gallium to suppress the rejection in a cardiac allograft transplantation mouse model, indicating that the survival of mice was significantly improved. Meanwhile, histological tests showed mitigated immune rejection. The other independent experiment also confirmed the immunomodulating potential of gallium in transplantation\textsuperscript{[105]}. 

Therefore, it is reasonable to further speculate that gallium can be applied as a unique metallic substance added to cryoprotectants before cryopreservation. When the organ is transplanted into the recipient, gallium of millimole level can rapidly affect the immune system, avoiding both violent inflammatory responses and grievous immune rejection, which are fundamentally dependent on the immunomodulatory effects of gallium on T cells and macrophages (Figure 6F). In addition, a large number of literature have also shown that gallium is a potent antibacterial substance compared
with traditional antibiotics in that pathogens have already become highly resistant to abused antibiotics, such as penicillin, whereas no drug resistance is observed after gallium treatment. The basic mechanism is still based on the competitive binding mechanism of gallium with iron essentially, which destroys the transport of iron ions and related metabolic processes in microorganisms, producing a great amount of ROS and further affecting microbial DNA, and protein synthesis[106,107].

The emerging LM-mediated cryo-biomedicine is an interdisciplinary field with the efforts and cooperation of material science, cryogenic engineering, and biomedicine. It is necessary to realize that LM-mediated cryo-biomedicine is still in its initial stage which challenges and opportunities coexist. Further development still requires efforts in underlying mechanism, material design and synthesis, as well as multi-scale regulation. A few issues still ask for further exploration: (1) LM-mediated synergistic effect is worth pursuing. The combination of LM and cryo-biomedicine provides a new approach in the face of challenges. Researchers should explore more potential applications of LM as well as LM composites. (2) Explore the application of liquid metals at nanoscales. Recently, the LM nanoparticles have been attracting increasing attention compared to their bulk counterpart due to the unique properties in heat and mass transfer. Thus, a more comprehensive understanding of LM nanoparticles in cryo-biomedical systems should be considered. (3) Achieving more precise regulation is desirable through improving LM preparation program and advanced modulating techniques. (4) The interface characteristics are of great significance for LM. Thus, tuning the proper interface between LM and biological systems is essential for its performance. Overall, the new rising direction holds broad prospect but also requires a joint effort from interdisciplinary contribution. And we do believe that LM will accelerate the development of cryo-biomedicine, and many breakthroughs will emerge with its promotion.
Figure 6. (A) LM enhances the imaging of CT and MRI in cryosurgery. (B) LM as an immune adjuvant for synergistic immune therapy. (C) Gallium ion-mediated cancer treatment through iron deprivation. (D) Strengthening heat transfer of cryopreservation through LM coating. (E) Mechanism of anti-inflammation of Ga$^{3+}$ by suppressing immune cell proliferation and promoting secretion of anti-inflammatory cytokines. (F) LM as an immunomodulating agent for relieving immune rejection.

DECLARATIONS

Author’s contribution

Proposed original conceptualization: Lu C, Yang F, Rao W

Outlined the manuscript structure: Lu C

Investigated literatures and wrote the original manuscript: Lu C, Yang F

Designed original figures: Yang F
Reviewed and revised the manuscript: Lu C, Rao W
Supervised the manuscript: Rao W

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Not applicable

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Not applicable

Consent for publication
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