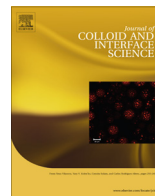




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Fluorescent magnetic nanoparticles for cell labeling: Flux synthesis of manganite particles and novel functionalization of silica shell



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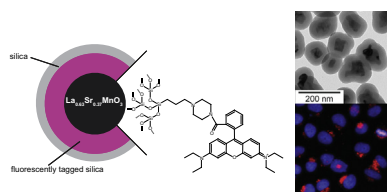
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GRAPHICAL ABSTRACT



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ABSTRACT

Novel synthetic approaches for the development of multimodal imaging agents with high chemical stability are demonstrated. The magnetic cores are based on $\text{La}_{0.63}\text{Sr}_{0.37}\text{MnO}_3$ manganite prepared as individual grains using a flux method followed by additional thermal treatment in a protective silica shell allowing to enhance their magnetic properties. The cores are then isolated and covered *de novo* with a hybrid silica layer formed through the hydrolysis and polycondensation of tetraethoxysilane and a fluorescent silane synthesized from rhodamine, piperazine spacer, and 3-iodopropyltrimethoxysilane. The aminoalkyltrialkoxysilanes are strictly avoided and the resulting particles are hydrolytically stable and do not release dye. The high colloidal stability of the material and the long durability of the fluorescence are reinforced by an additional silica layer on the surface of the particles. Structural and magnetic studies of the products using XRD, TEM, and SQUID magnetometry confirm the importance of the thermal treatment and demonstrate that no mechanical treatment is required for the flux-synthesized manganite. Detailed cell viability tests show negligible or very low toxicity at concentrations at which excellent labeling is achieved. Predominant localization of nanoparticles in lysosomes is confirmed by immunofluorescence staining. Relaxometric and biological studies suggest that the functionalized nanoparticles are suitable for imaging applications.

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1. Introduction

Medical and biological applications of magnetic nanoparticles have been extensively investigated over the past two decades. Such particles can be effectively employed as contrast agents in mag-

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netic resonance imaging (MRI), not only for diagnostic purposes in human medicine but also in biological research for cell labeling and tracking *in vivo* [1,2]. Similarly, the results of early clinical trials of the use of magnetic nanoparticles in cancer treatment based on magnetically induced hyperthermia are also promising [3]. Furthermore, magnetic nanocarriers offer multiple advantages in bio-separation [4,5] and may provide a vector for targeted drug delivery [6]. With the emergence of more complex nanomaterials that integrate several functional modalities at the nanoscopic level, completely new applications and hybrid techniques have become available. Tremendous progress in bioimaging has been achieved by virtue of multimodal agents that enable the combination of visualization *via* MRI with optical imaging [2,7], imaging based on radioactive isotopes (PET, SPECT) [8], or ultrasound imaging [9].

Various materials have been investigated as prospective magnetic cores for bioapplications, namely, ferro- or ferrimagnetic oxide phases, most commonly iron oxides, *i.e.*, maghemite and magnetite, although ferrites [10,11] and manganites [12–14], *etc.* have been also studied. Iron oxide nanoparticles are of particular interest, primarily because of their low toxicity and facile synthesis as well as the extensive available knowledge regarding the biological fate of these particles. However, it has been demonstrated that multicomponent oxides may allow for better tuning of magnetic properties for particular applications compared with what can be achieved using simple binary compounds such as iron oxides [15]. An excellent example is the perovskite $\text{La}_{1-x}\text{Sr}_x\text{MnO}_3$ (LSMO) phase, the Curie temperature of which can be adjusted through modification of the composition and size of the particles to a level suitable for use in the magnetically induced hyperthermia [16]. Upon exceeding the Curie temperature, the particles no longer dissipate energy of an AC magnetic field, and thus, the risk of local overheating is ruled out. Moreover, the high susceptibility of LSMO particles indicates that this material could be useful for a variety of other applications. Recently, a dual MRI/fluorescence contrast agent based on LSMO nanoparticles has been tested for cellular labeling [1,17], and LSMO nanocarriers have been employed for the separation of DNA [4].

LSMO nanoparticles are traditionally prepared *via* various sol-gel methods using citric acid and ethylene glycol (Péchine process) [12,16], amino acids [13,18], or polyvinyl alcohol [19] as the gelifying agents. Unfortunately, single-phase manganite is obtained only after the complex sol-gel precursor is heated to an elevated temperature, typically higher than 650 °C. Consequently, partial sintering begins to occur in the product, and connecting bridges form between grains [20,21]. It is possible to break these connections through harsh mechanical processing. However, such treatment is accompanied by a broadening of the size distribution and the formation of a fraction of very fine fragments [21]. Several other synthetic procedures for LSMO nanoparticles have been reported, namely, the hydrothermal method [22,23], aerosol flame pyrolysis [24], and flux preparation, the so-called molten salt synthesis (MSS) [25,26], but only the last one seems to overcome the drawbacks of sol-gel routes with respect to the morphology and size distribution of the particles. However, LSMO nanoparticles prepared *via* MSS have not yet been tested in highly demanding applications such as those in biology or medicine, and existing reports address solely their magnetic properties and structure [27,28]. In particular, these studies have revealed rather low magnetic moments and reduced Curie temperatures of the as-prepared MSS nanoparticles, but at the same time, it has been demonstrated that the magnetic properties of these particles might be considerably enhanced by post-synthetic annealing (henceforth referred to post-annealing). To prevent the growth of manganite cores and the occurrence of sintering processes at elevated temperature,

post-annealing must be applied after silica coating. Afterwards, the manganite nanoparticles can be easily released through alkaline dissolution of the silica components [27,28]. Hence, post-annealing in silica allows the advantages of flux synthesis to be maintained while improving the magnetic properties of the products.

This contribution reports on the synthesis of a bimodal MRI/fluorescence imaging agent with magnetic cores based on $\text{La}_{0.63}\text{Sr}_{0.37}\text{MnO}_3$ manganite prepared *via* MSS followed by post-annealing in silica nanoreactors. The magnetic cores are isolated and coated *de novo* with a hybrid silica shell, which incorporates covalently attached rhodamine and exhibits enhanced chemical stability by virtue of its novel aminoalkyltrialkoxysilane-free synthesis. Often, silica layers with covalently attached organic moieties are prepared through the organic derivatization of aminoalkyltrialkoxysilanes, such as 3-aminopropyltriethoxysilane (APS), that are subsequently employed in the coating of particles or for terminal grafting of organic molecules onto their silica surfaces. Alternatively, hybrid silica shell containing aminoalkyl moieties is prepared at first and specifically functionalized afterwards. However, it has been indicated that complex nanoparticles obtained by these traditional procedures show some hydrolytic instability: the dye, although covalently embedded in a silica shell, leaches out of the particles, or an organic corona, originally attached to the particle surface, is gradually released [29–33]. A clear evidence for the hydrolytic deterioration of such organically functionalized nanoparticles was published as early as 1980 [29]. Although systematic studies dealing with the chemical stability of organic functionalization and the specific role of aminoalkyltrialkoxysilanes are very scarce, the underlying mechanism of the hydrolytic instability is not entirely unclear. Bridger and Vincent [29] originally suggested three possible processes that caused the deterioration of their PEGylated nanoparticles: the hydrolysis of the urethane link employed for the attachment of poly(ethylene oxide) to APS, the oxidative degradation of the poly(ethylene oxide) chains, and the dissolution of surface silica layers. The last process is particularly relevant when residual free amino groups are present in the silica shell since the hydrolysis of siloxane bond is a base-catalyzed reaction. Silica matrices containing APS are thus especially prone to hydrolytic processes. Thorough studies of aminopolysiloxanes prepared by the polycondensation of APS and tetraethoxysilane (TEOS) were undertaken by Zhmud and Sonnefeld who evidenced high solubility in water and gradual release of the organic component [34]. Conclusive quantitative data on the stability of materials obtained by grafting APS onto silica were also provided by Etienne and Walcarius [33].

Based on these reports we realized that incomplete conversion of amino groups during the organic functionalization of aminoalkoxysilanes or poor purification of the resulting complex silanes may significantly reduce the hydrolytic stability of the final nanoparticles. However, the type of bonding involved in hybrid silica shells should be also taken into account. The stability of both the hybrid shell and surface anchoring might be seriously impaired if the condensation of alkoxysilanes does not dominate and only adsorption *via* multiple hydrogen bonds occurs instead. This non-covalent adsorption can be easily recognized by instantaneous leaching during purification of the final product and can be prevented by suitable adjustment of reaction conditions [35].

To avoid the use of any aminoalkylsilanes, our synthesis is accomplished through the alkylation of the fluorophore with 3-iodopropyltrimethoxysilane, leading to a complex silane that is isolated and subsequently used in the silica coating procedure of the Stöber's type. The preparation of the fluorescent particles is completed with the application of an additional layer of pure silica

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