BRIEF REPORT

REVISED Fabrication of hydrogel mini-capsules as carrier systems

[version 3; peer review: 3 approved, 2 approved with reservations]

Elisa Roberti[1,2*], Gaia Petrucci[1,2*], Francesco Bianciardi[1,2], Stefano Palagi[1,2]

[1]The BioRobotics Institute, Sant'Anna School of Advanced Studies, Pisa, Tuscany, 56025, Italy
[2]Department of Excellence in Robotics & AI, Sant'Anna School of Advanced Studies, Pisa, Tuscany, 56127, Italy

* Equal contributors

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Abstract

Conventional drug administration often results in systemic action, thus needing high dosages and leading to potentially pronounced side effects. Targeted delivery, employing carriers like nanoparticles, aims to release drugs at a target site, but only a small fraction of nanoparticles reaches it. Microrobots could overcome this issue, being guided to hard-to-reach sites and locally delivering payloads. To enhance their functionality, we propose microrobots made as deformable capsules with hydrogel shells and aqueous cores, having the potential added advantages of biocompatibility, permeability, and stimulus-responsiveness. Endowing microrobots with deformability could allow them to navigate inside capillaries and cross barriers to finally reach the target site. In this study, we present a cost-effective method for fabricating core-shell structures without the use of organic solvents, surfactants, or extreme pH conditions. First, a mixture of hydrogels, agarose and alginate, is dripped into a calcium chloride solution to form beads. After they are loaded with calcium ions at different concentrations, they are immersed in an alginate solution to form the shell. Finally, the beads are heated to let the agarose melt and diffuse out, leaving a liquid core. By varying the concentration of calcium ions, we obtain shells of different thicknesses. We have correlated the measured shell thickness to its colour intensity and extrapolated to estimate the thickness of shells too thin to be measured directly. This allowed us to conclude that no continuous shells forms below a certain calcium chloride concentration. For higher concentrations, although the core may

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1. **Mahmut Selman Sakar**, Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland
2. **Dr. Sudhir G. Warkar**, Delhi Technological University, Delhi, India
3. **Samuel Stealey**, Saint Louis University, Saint Louis, USA
4. **Bradley Nelson**, Multi-Scale Robotics Lab, Zurich, Switzerland
   Fabian Landers, ETH Zurich, Zürich, Switzerland
5. **Min-Soo Kim**, ETH Zurich, Zurich,

remain partially gelled, continuous shells successfully form. To qualitative assess core-shell capsule deformability, we forced them through a tube with an inner diameter ~1.6 times smaller than the average capsule diameter. The capsules deformed to pass through the constriction while maintaining structural integrity. Therefore, our fabrication method offers a promising platform for applications in drug delivery, encapsulation systems, and microrobotics.

Plain language summary

Conventional drug administration methods often lead to widespread effects in the body and dosage-related side effects. Targeted drug delivery aims to release drugs precisely where they're needed by using carriers. However, only a few of them actually reach the intended target. To address this issue, scientists are exploring microrobots, tiny machines that can reach places deep inside the body. We design microrobots as soft liquid-filled capsules. In our research, we've developed an affordable method to make these capsules without using harsh chemicals. The obtained capsules have a controllable shell thickness and a liquid or almost liquid core. This makes our capsules promising candidates as microrobot bodies or as drug delivery carriers.

Keywords

alginate, agarose, core-shell, hydrogel capsules, microrobotics

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Corresponding authors: Elisa Roberti (elisa.roberti@santannapisa.it), Stefano Palagi (stefano.palagi@santannapisa.it)

Author roles: Roberti E: Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Petrucci G**: Data Curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Bianciardi F**: Conceptualization, Investigation, Writing – Original Draft Preparation; **Palagi S**: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Methodology, Project Administration, Software, Supervision, Validation, Visualization, Writing – Review & Editing

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REVISED **Amendments from Version 2**

The Abstract and Introduction sections have undergone minor revisions.

To address some of the reviewers' questions, we conducted deformability experiments, which are now described in Section 2.3.3 of the Methods chapter. The results of these experiments are presented in Section 3.3 of the Results chapter, where we have also included Table 1, summarizing the diameters of the tested capsules and the number that successfully passed the deformability test.

In light of these additions, we have introduced two new figures: Figure 4 and Figure 7.

Significant revisions have also been made to the Conclusion. Furthermore, five new references have been added to the Introduction section.

The Underlying Data and Extended Data have also been updated. Specifically, the Extended Data now include:

- a time-lapse video showing the release of green dye from core-shell capsules,

- a video demonstrating the deformability test procedure, and

- an image of core-shell capsules produced using low-melting agarose.

Additionally, we have reorganized the Underlying Data, adding a utility script (_init_jl) to help reproduce the computational environment.

Any further responses from the reviewers can be found at the end of the article

1 Introduction

Most routes of drug administration (*e.g.* intravenous) lead to systematic action of the drug, as local administration is possible only for suitable diseases. In many cases, this requires much higher dosages than those needed if the administration could be local, resulting in much more significant side effects[1]. This is particularly problematic for chemotherapy. To address this issue, conventional drug delivery approaches aim to improve aqueous solubility and chemical stability of the drug, increase its pharmacological activity and reduce side effects while maintaining the therapeutic concentrations at the target site[2]. A suitable approach to reduce drugs' side effects consists in adopting carriers, such as nanoparticles, that could release the drug only at the target site or to target tissues or cells, which is known as targeted drug delivery[3,4]. The drug loading in nanosystems could be categorized into three main strategies: pre-loading, co-loading, and post-loading. Pre-loading involves forming drug nanoparticles first and then coating them with a stabilizing shell. Co-loading, the most common method, incorporates the drug during the nanocarrier's while post-loading, introduces the drug into already formed nanocarriers[5]. Whereas nanoparticles-based targeted drug delivery has proved to enhance the therapeutic index and reduce adverse side effects, there are still open issues related to drugs toxicity, selectivity and dosage. One key problem is that only a tiny fraction of the drug-loaded nanoparticles reaches the target site (e.g. a solid tumour)[6].

Microrobots have been proposed to overcome these issues[7]. The main feature of these tiny robotic devices is their controlled navigation, which could allow them to be guided to hard-to-reach target sites inside the human body. Microrobotics could thus become a non-invasive approach to locally administer drugs (or drug-loaded carriers) at sites that are not currently suited to local administration. Although recent advances in microrobotics for drug delivery applications, tracking and guiding microrobots *in vivo* remains challenging, and their ability to move through body tissues and cross biological barriers is still limited[7]. To make microrobots ultra-deformable and able of moving across tissues and barriers, a possible design consists of a thin, soft shell enveloping a liquid core[8,9]. Ultra-deformability refers to the ability of these microrobots to deform substantially and reversibly allowing them to pass through openings[10–13]. We take inspiration from leukocytes, particularly neutrophils and macrophages, which are sufficiently deformable to move inside small capillaries (< 5 μm) that reach solid tumours, travel through the interstitial spaces of tissues (< 1 μm), and extravasate through hundreds-nanometres junctions between cells of the leaky vasculature in the tumour microenvironment[6]. Moreover, the microrobots' building materials should be biocompatible or biodegradable. Additionally, the shell should be permeable and possibly stimuli-responsive, releasing.

Core-shell and hollow particles can be prepared using a variety of methods[14,15], including: Layer-by-Layer (LbL)[16,17], sol-gel[18,19], solvent evaporation, spray-drying[20], double emulsions (O/W/O or W/O/W)[21]. However, none of these methods is suited to the fabrication of thin-shell gel-based microrobots we have conceived, which requires mild process conditions to avoid damages to the potential payloads.

In this article, we introduce a method for fabricating core-shell capsules that have a liquid aqueous core and a hydrogel shell, which involves no organic solvents, surfactants, or low/high pHs. We exploit the different gelation mechanisms of alginate and agarose to create the core-shell capsules. Specifically, alginate crosslinks in the presence of calcium ions, while agarose gelate at room temperature. We report the characterization of the obtained core-shell capsules, with a focus on the shell thickness and its dependence on the process parameters. We also qualitatively evaluated the deformability of the core-shell capsules by forcing the capsules through a silicone tube. The proposed process holds potential also for the fabrication of micro-capsules and core-shell particles for different applications, including drug-delivery systems other than microrobotics ones.

2 Methods

2.1 Materials

Sodium alginate (CAS 9005-38-3), agarose (CAS 9012-36-6), sodium citrate dihydrate (SCD)(CAS 6132-04-3), calcium chloride (CAS 10043-52-4) and iron (III) oxide nanopowder <50 nm (CAS 1309-37-1) were purchased from Merck. 0.45 μm filters (CAS 514-1267) were purchased from VWR®.

silicone tube (CAS 667-8441) was purchased from RS PRO. 10 mL Syringes (CAS MDSS10SE) were purchased from Terumo®. MilliQ water is used unless differently stated (Elix® Advantage 10 Water Purification System).

To fabricate the shell we have chosen alginate, a natural polymer which shows attractive properties such as biocompatibility, low cost, ease of gelation, inert nature[22]. Alginate undergoes a reversible gelation process when divalent ions (*e.g.* Ca^{2+}, Ba^{2+} or Sr^{2+}) are added to the polymeric solution and it returns to the original liquid form when exposed to chelating agents that bind the divalent ions with a higher affinity constant[23].

2.2 Preparation of the core-shell particles

The process for preparing the core-shell beads is depicted in Figure 1 and a tutorial video can be found in the Extended Data[24].

1. The agarose-alginate water solution is prepared mixing a 1% w/v agarose solution (previously heated at 95°C to promote dissolution) and a 4% w/v alginate solution to have overall concentrations of 0.5% agarose and 2% alginate. The obtained warm solution is filtered (0.45 µm filters) and then it is added drop-wise to a $CaCl_2$ 100 mM solution using a 10 mL syringe (Terumo®) to form gel beads because of the immediate crosslinking of alginate by calcium ions. The beads are kept in the $CaCl_2$ solution for about 10 minutes to complete the crosslinking.

2. The beads are then washed with 100 ml of water to remove the excess of $CaCl_2$, transferred into a 50 mM solution of sodium citrate dihydrate (SCD) and kept overnight. SCD is meant to chelate Ca^{2+} ions and thus de-crosslink the alginate template, leaving beads of just agarose.

3. The agarose beads are then washed again with water and transferred in $CaCl_2$ solutions at different concentrations (ranging from 0.1 mM to 100 mM).

4. After 1 hour of equilibration in the $CaCl_2$ solution, the beads are picked up and briefly placed on a filter paper to remove the excess solution from the surface. They are then immersed for 15 minutes in a solution of alginate 0.5% w/v to form an alginate shell around the agarose bead templates. The alginate solution contains 0.2 mg/ml of 50 nm Fe_2O_3 nanoparticles to enhance the shell visualization under the optical microscope.

5. After 15 minutes, the core-shell structures are briefly rinsed in water and then transferred in a 100 mM $CaCl_2$ solution to complete the crosslinking of the alginate shell.

6. To obtain a liquid core, the core-shell structures are placed in a thermal bath at a temperature of 90/95°C for 2–7 hours, allowing the agarose to liquefy and diffuse out, leading to microcapsules with an alginate shell and a liquid aqueous core.

We immersed capsules purposely made using cores loaded with particles ($CaCO_3$ or SiO_2) in a SCD solution to de-crosslink the shell and assess the core liquidity. The release of the particles from the capsules highlighted that the cores were insubstantial after 6–7 hours of thermal bath, although we still observed a light gel residue (see Extended Data, video "ChargedCapsuleSiO2_core")[24].

2.3 Capsules characterization

2.3.1 Shell formation and thickness

We characterized the beads using an Hirox optical digital microscope (Hirox HRX-01) and analysing the images by two different approaches: i) direct measure of the shell thickness – this method is however challenging for thin shells; ii) indirect measure based on shell colour.

We assume the volume V_s, and thus the thickness of the alginate shell, to be proportional to the amount of Ca^{2+} ions loaded into the core (see Figure 2), such that

$$V_s = \alpha \, n_{Ca^{2+}} \tag{1}$$

where α is a proportionality constant (to be determined) and $n_{Ca^{2+}} = V_c [Ca^{2+}]$ is the number of Ca^{2+} ions moles in

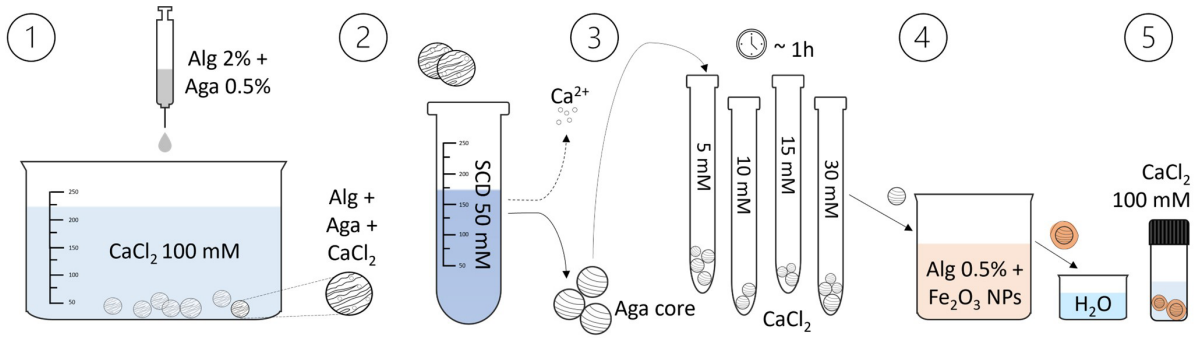

Figure 1. Illustration of the core-shell particles fabrication process. The core-shell beads preparation involves the dripping of an agarose-alginate mixture in a $CaCl_2$ solution, followed by washing and transferring the beads to a sodium citrate dihydrate (SCD) solution. After loading them with Ca^{2+} ions (by equilibration in $CaCl_2$ solutions of different concentrations), the beads are placed in an alginate solution containing Fe_2O_3 nanoparticles to form the shell. Subsequently, they are washed and transferred to another $CaCl_2$ solution to consolidate the shell. By heating the beads, the agarose liquefies and diffuses out, resulting in a liquid core within the shell.

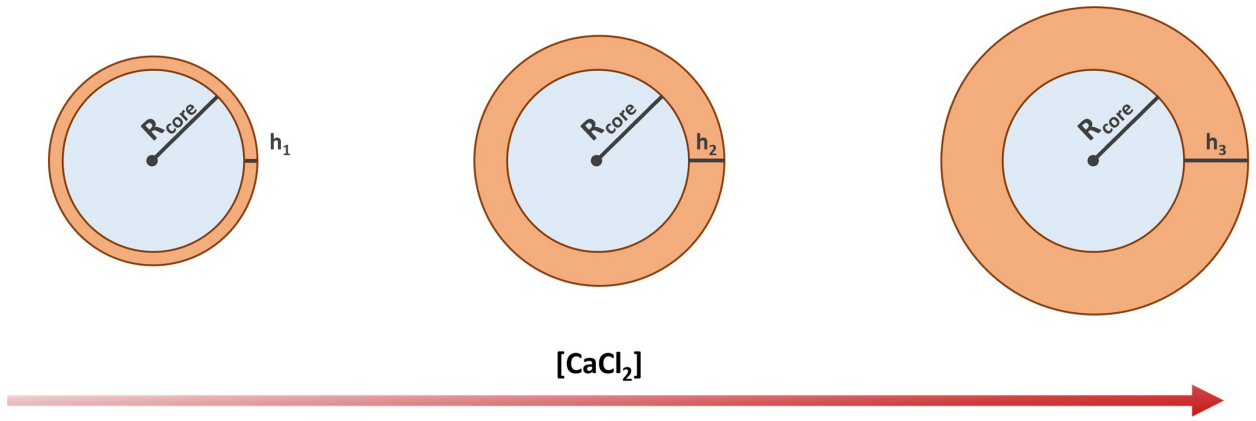

Figure 2. Schematics showing the expected shell thickness increase with the increasing concentration of calcium chloride.

the core ($[Ca^{2+}]$ is assumed to be equal to the concentration of the $CaCl_2$ solution the cores have been soaked in before forming the alginate shell). Considering $V_c = \frac{4}{3}\pi R_c^3$ (where R_c is the core radius) and $V_s = \frac{4}{3}\pi[(R_c + h)^3 - R_c^3]$ (where is h the thickness of the shell), we obtain

$$h = R_c\left(\sqrt[3]{\alpha[Ca^{2+}]+1} - 1\right) \quad (2)$$

When the core is immersed in a solution at a fixed alginate concentration, the release of Ca^{2+} ions induces the crosslinking of alginate around the cores. This produces a shell whose thickness depends on the number of Ca^{2+} ions previously absorbed by the core. Therefore, we assume n Ca^{2+} is and thus to V_s. We then fabricated batches of capsules by soaking the agarose cores in $CaCl_2$ solutions at concentrations ranging between 0.1 and 100 mM (0.1, 1, 2, 5, 10, 15, 20, 30, 50 and 100 mM, three to five samples for each concentration) – see Figure 3. We stained the shell by adding iron oxide nanoparticles to the alginate solution to enhance its detection and ease the measurement of its thickness (particles were chosen instead of a dye, as most dyes would diffuse away and/or stain the core too).

We first measured the shell thicknesses using the measuring tool of the digital optical microscope's software. To perform a linear fit and obtain an estimate for α, we rearranged Equation 2 to obtain an equation of the form $y = \alpha x$, leading to:

$$\left(\frac{h}{R_c} + 1\right)^3 - 1 = \alpha[Ca^{2+}] \quad (3)$$

Once obtained a value for α, Equation 2 can be used to calculate the expected shell thickness as a function of the $CaCl_2$ solution concentration. Therefore, we estimated the thickness of the shells too thin to be directly measured by extrapolating values from the fitted curve.

To allow for estimating the thickness of such thin shells from actual images of the beads, we correlated the shell thickness to its colour intensity (given by the iron oxide nanoparticles). To have consistent measurements of the colour intensity, we set and kept fixed the parameters for the microscope images acquisition (illumination, camera settings). The parameters values were chosen to obtain good quality images with all the inspected thicknesses. The image processing develops as follow:

1. each image of the set is segmented creating two clusters: one with all the pixels corresponding to the capsules, and a second one with the background;
2. in each image, a small portion at the centre of the capsule cluster is automatically selected, and the RGB values of the corresponding pixels are extracted;
3. in each image, an average value for each of the three colour channels is calculated over the selected region;
4. in each image, average channel values corresponding to the background are also calculated;
5. from the colour intensities extracted at point 3 and 4, the red channel value, the value of the red channel minus the background, and the value of the red channel minus the blue channel are calculated and related to the $CaCl_2$ concentration and to the theoretical shell thickness (calculated with Equation 2);
6. a linear fit of the measured shell thickness over the measured R–B colour intensity is performed, obtaining a relation to estimate the shell thickness from the colour intensity.

From this we again extrapolate the expected thickness for the non-measurable shells and compare the predictions with those obtained from Equation 2.

2.3.2 Core dissolution

To ensure good degree of deformability, our core-shell capsules should consist of a highly soft shell and a predominantly liquid core, as previously mentioned. Step 2 and step 6 of

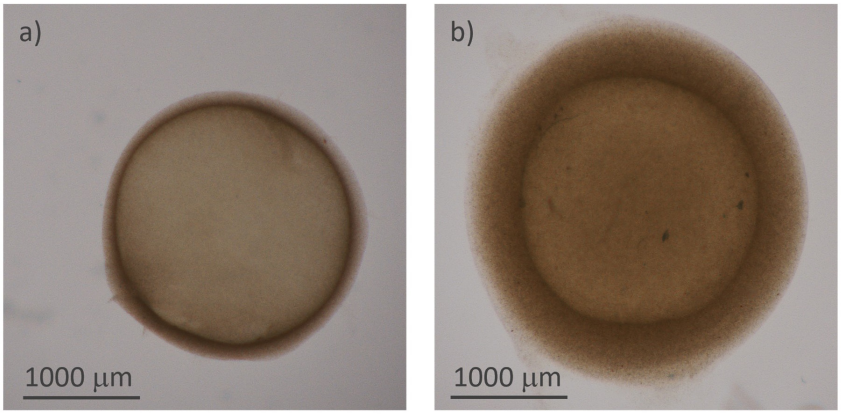

Figure 3. Structure of the hydrogel microcapsules: **a**) and **b**) optical microscope images of two capsules of different thicknesses.

the fabrication protocol are indeed aimed at removing the alginate and the agarose, respectively, from the core. Step 6, in particular, aims at melting the agarose and letting it diffuse out through the alginate shell, ideally leading to a completely liquid core. To assess the consistency of the core, we loaded the capsules with $CaCO_3$ or SiO_2 particles and observed their release from the core. $CaCO_3$ particles were nucleated directly in the preformed cores through the precipitation reaction of $CaCl_2$ and Na_2CO_3, after step 2 of the protocol, by immersing the cores in a 0.33 M solution of $CaCl_2$ and then adding under mixing a 0.33 M solution of Na_2CO_3, thus reaching supersaturation conditions and leading to the sudden precipitation of $CaCO_3$ particles. Instead, SiO_2 microparticles were loaded in the alginate/agarose solution before forming the cores at step 1 of the protocol.

In both cases, we dissolved the shell by placing the capsules into a 50 mM SCD solution to de-crosslink the shell. We then observed at the optical microscope the release of the loaded particles to determine whether the core was fully liquid or not (see Extended Data, video "ChargedCapsuleSiO2_core")[24].

2.3.3 Deformability tests

To qualitatively evaluate the deformability of our core–shell capsules, we conducted tests on two types of capsules: capsules with ideally liquid cores, obtained through thermal treatment at 95 °C, and core-shell particles with solid cores, produced by omitting the final heating step (see step 6 in Section 2.2). The test consists of forcing these capsules through a silicone tube with an internal diameter of 1.6 mm using a syringe (see Figure 4). Specifically, we tested a few representative capsules per type with a shell composed of alginate crosslinked with 30 mM and 50 mM $CaCl_2$ and with a diameter larger than the inner diameter of the silicone tube. The capsules were drawn into the tube using a 10 mL syringe, and their passage through the tubing was observed using a digital optical microscope (Hirox HRX-01).

3 Results & discussion

3.1 Characterization of the capsules – shell thickness

The protocol described in Section 2.2 led to the successful fabrication of the microcapsules. Figure 3 reports the microscopy images of two representative samples, acquired using transmitted illumination to emphasize the shell and measure its thickness.

First, in Figure 5(a), the data related to the measured thicknesses of the hydrogel capsules are linearized through the left side of Equation 3 and plotted against $CaCl_2$ concentration (which equals Ca^{2+} concentration). Fitting the right side of Equation 3 to the linearized thickness data, we found a value of 0.033 mM^{-1}. In Figure 5(b) the comparison between the thickness values predicted by the fitted Equation 3 (orange crosses) and the measured ones (blue dots) is shown to be consistent. The fitted equation also provides a first estimation of the shell thickness for $[CaCl_2] < 5$ mM, for which we could not measure the thickness directly.

As explained in Section 2.3, we extracted the average intensity of the red, green and blue components from an area corresponding to the centre of the capsules in the acquired images. Considering the reddish colouring given by iron oxide nanoparticles to the shell, we have chosen the red component, red minus background, and red minus blue colour intensities to be plotted against the concentration of $CaCl_2$ soaked by the core of each particle (Figure 6(a)).

Figure 6(a) shows that the values obtained as the difference between the red and the blue components (R−B) do not saturate at high $[CaCl_2]$, which instead happens in the other two cases. Therefore, we chose the R−B combination as the most reliable to be correlated to the shell thickness. In Figure 6(b), the measured R−B colour intensity is plotted against the theoretical shell thickness calculated by Equation 2. The plot is non-linear, with a threshold for low thickness values. The

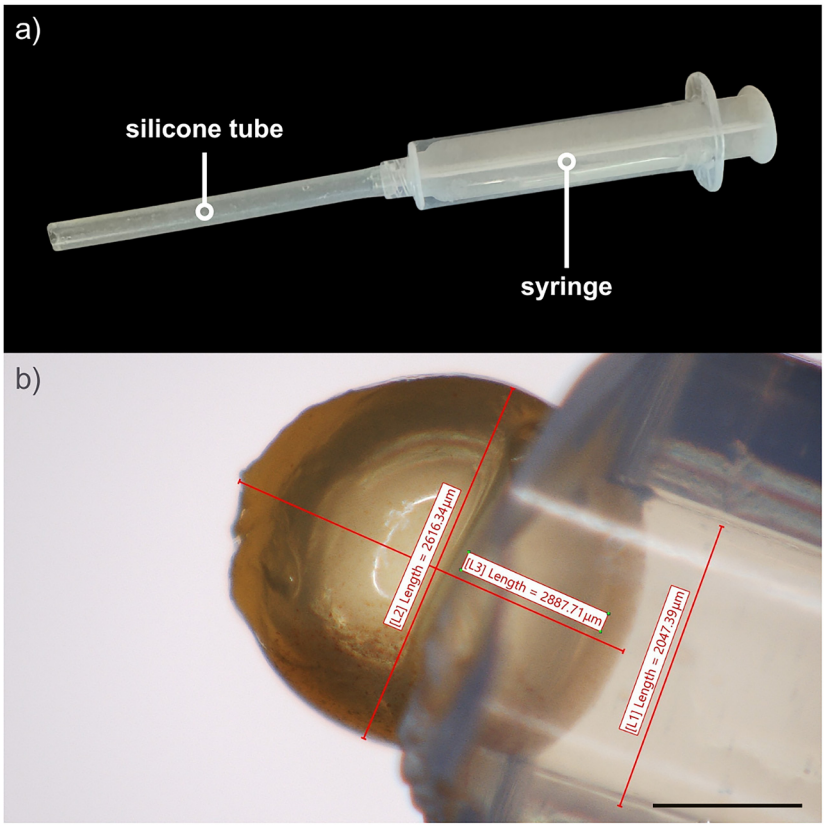

Figure 4. Qualitative deformability tests. a) experimental setup used to assess the deformability of core-shell capsules. It consists of a silicone tube with an internal diameter of 1.6 mm connected to a plastic syringe. The capsule is drawn from the open end of the tube opposite to the syringe; **b)** the internal diameter of the tube is smaller than the capsule size. Scale bar: 1000 µm.

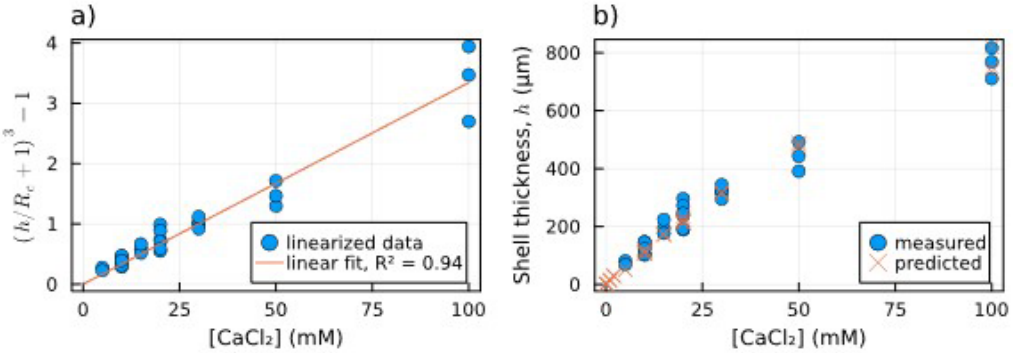

Figure 5. Dependence of shell thickness on [Ca^{2+}]: **a**) Linearized measured shell thickness (blue dots) alongside the linear fit of the data (orange line); **b**) Comparison between measured shell thickness (blue dots) and the thickness estimated using the parameter obtained from the fitting (orange crosses).

measured shell thickness values are plotted against the calculated R–B colour intensity, and the linear correlation between these two data was verified and calculated through a linear fit (Figure 6(c), blue dots and orange line). The crosses lying on the left side of the fitting line correspond to the capsules for which the shell thickness was not measurable. The linear relation leads to the prediction of negative thickness values, which are obviously not possible, confirming our hypothesis of a

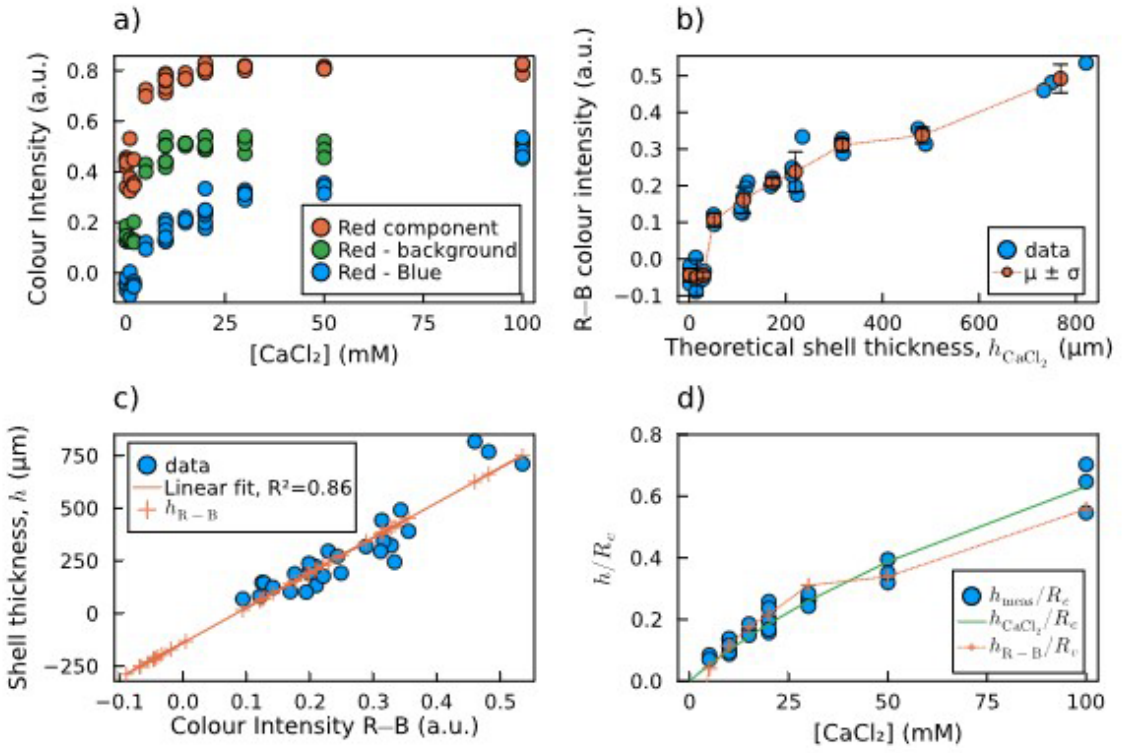

Figure 6. Estimation of shell thickness via colour analysis: **a**) Plot of the mean colour intensity in the selected area of each image: values of the red component (R, red dots), the red minus the background (R–bg, green dots), and the red minus the blue component (R–B, blue dots). **b**) Calculated R–B colour intensities and their mean and standard deviation for each concentration of calcium chloride *vs* the theoretical shell thickness calculated by Equation 2. **c**) Direct linear correlation between measured shell thickness and R–B colour intensity, including thickness predictions for both measured and non-measured sample. For the latter samples, the fitting predicts negative thickness values, which are not possible. **d**) Comparison of normalized shell thickness: measured (blue dots), predicted by Equation 2 (green solid line), and predicted by the R–B colour intensity (orange crosses and dotted line).

threshold behaviour. This means that below a certain core [$CaCl_2$] threshold, alginate shells did not form on top of the agarose cores.

Figure 6(d) shows the comparison between the two estimations and the measured thickness as normalized shell thickness vs concentration of $CaCl_2$ soaked by the core. They are in good agreement, except for the points below 5 mM, for which the R–B colour intensity hints to the absence of a shell, which makes this second method more reliable for detecting the shell thickness rather than a simple linear extrapolation.

All images, data, and analysis scripts can be found in the Underlying Data[25].

3.2 Characterization of the capsules – core dissolution

The tests conducted with SiO_2 and $CaCO_3$ nanoparticles within the core of our capsules, were designed solely to evaluate the dissolution of the core.. Theyindicate that the core does not fully liquefy, likely because some agarose does not completely diffuse out. Specifically, when the capsules were placed back in the SCD solution after step 6, it was assumed that the agarose had melted due to the thermal bath. Under these conditions, we expected to observe the gradual release of SiO_2 particles as the shell dissolved. Although this release does occur, a residue of gel, likely agarose, appears to persist (see Extended Data, video "ChargedCapsuleSiO2_core")[24].

3.3 Deformability test

Deformability tests revealed that the core-shell capsules fabricated using the proposed protocol are highly deformable, even though the core is not fully liquid. We evaluated two capsules with a shell composed of alginate crosslinked with 50 mM $CaCl_2$ and two with a shell crosslinked with 30 mM $CaCl_2$. For comparison, we also tested the deformability of four core-shell particles with a solid core – i.e., not subjected to step 6 of the protocol – while maintaining the same shell composition: two with a shell crosslinked with 30 mM and two with 50 mM $CaCl_2$). Following passage through the constriction, three out of the four capsules subjected to step 6 (core dissolution) retained their structural integrity under both shell conditions (see Figure 7b), as reported in Table 1. However,

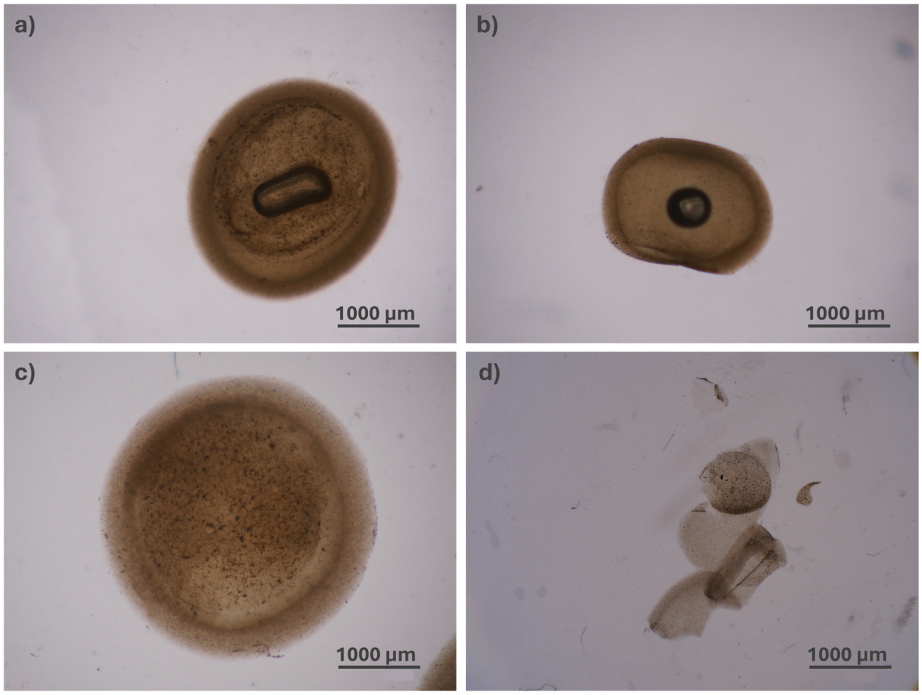

Figure 7. Capsules before 1 **a**), **c**) and after **b**), **d**) the passage through the silicone tube. The core-shell capsule with a semi-liquid core composed of a shell made of alginate crosslinked with 50 mM $CaCl_2$, shown before **a**) and after **b**) passage through the silicone tube. The capsule clearly deforms to pass through the silicone tube while maintaining the integrity of their structure. In contrast, the core-shell particle with a solid core completely lost its structural integrity after passing through the silicone tube (Figure 7d).

Table 1. Dimensions of core-shell capsules and particles, and outcome of the deformability test. The table reports the diameters of the tested core-shell capsules and the corresponding outcome after passing through the silicone tubing. In case #1, the capsule broke during the drawing phase, prior to undergoing the actual deformability test.

	Core-shell capsules (semi-liquid core)			
	30mM		50mM	
	#1	#2	#1	#2
Diameter (µm)	1295	1297	2043	2056
Survived	no	yes	yes	yes

in one of the two capsules with a shell crosslinked at 30 mM, capsule rupture occurred during syringe drawn. In contrast, all solid-core particles were larger than semi-liquid core ones and failed to maintain their structural integrity.

4 Conclusion

Most drug administration methods require high dosages and cause significant side effects, especially chemotherapy. A promising solution lies in drug-delivery systems (e.g. nanosystems) where the drug is encapsulated to reduce both dosage and toxicity, while also enabling targeted release. Among these systems, nanoparticles are widely used. However a major limitation is that, because administration is usually systemic, only a small fraction (<1%) of the administered dose typically reaches the target site. Microrobots may offer a solution by enabling precise navigation to hard-to-reach areas in the body and, thus, local administration of drug-loaded nanoparticles. Making microrobots ultra-deformable microrobots could help reach such areas and cross biological barriers. A promising design for such microrobots includes a thin, soft shell surrounding a liquid core. In this work, we presented a method for fabricating capsules with a soft hydrogel shell and a liquid aqueous core as a preliminary step toward the realization of ultra-deformable microrobots. The method uses sacrificial agarose cores as a template to obtain alginate shells of different thicknesses and liquid or insubstantial cores. Through the encapsulation of microparticles and the subsequent dissolution of the shell, we observed that the resulting core is not completely liquid, yet insubstantial

enough not to considerably affect the deformability of the capsules. This was further demonstrated by passing one representative capsule through a silicone tube with a diameter smaller than that of the capsule. Following passage, the capsule maintained its structural integrity demonstrating its ability to undergo significant deformation.

We also correlated the thickness of our capsules' shell from microscopy images to their colour intensity. This was especially meant to estimate the shell thickness of capsules with a shell that could not be directly recognized and measured from microscope images. By these observations, we concluded that calcium chloride solutions of concentration ≤2 mM lead to capsule shells that are discontinuous or not forming at all instead of the thin shells we expected from Equation 2. While we cannot definitively rule out the presence of an alginate shell, we strongly suspect that its formation is minimal or incomplete and, in either case, inadequate for the intended purpose of this study.

Our fabrication method allows to make capsules with a shell thickness h down to ~0.1× R_{core}. Therefore, by miniaturizing the sacrificial cores to the tens of microns range, we expect to obtain much thinner yet continuous shells (in the microns range). Indeed, the assessment of the fabrication method is performed on millimetre-sized capsules to ease the process and analysis, and further effort would be needed to adapt this method to make micron-sized capsules. The miniaturization of the capsules is primarily limited by the use of syringe needles for the initial formation of the sacrificial cores. For this reason, alternative methods that enable the production of smaller cores (e.g. microfluidics or spray techniques) should be investigated. Nevertheless, even if micrometric capsules are successfully produced, considering the realistically attainable shell thickness and contingent upon an assessment of their deformability, they might be able to move through channels (e.g. capillaries) smaller than their diameter, yet a concern remains as to whether these core-shell microcapsules could pass through junctions at the hundreds-of-nanometres scale. Indeed, while leukocytes and other cell types possess membranes with thicknesses on the order of nanometres, it appears that our current method cannot achieve nanometric shell thicknesses in tens-of-micron capsules.

In view of future applications, depending on the specific drug to be delivered, adaptations to the fabrication protocol may be required, for example, the use of low-melting-point agarose to avoid exposure to elevated temperatures that could compromise the stability of thermosensitive cargoes. We envision ultra-deformable microrobots capable of moving through body tissues and locally administering smart nanomedicines. Such administration could be triggered by external inputs or specific local conditions, e.g. altered pH. Although there are challenges to overcome, the development of ultra-deformable microrobots represents a promising approach to enhancing the efficacy and precision of drug delivery systems in biomedical applications.

Data availability

Underlying data

Zenodo: Fabrication of Hydrogel Mini-Capsules as Carrier Systems - Underlying data. https://doi.org/10.5281/zenodo.8200188[25].

This project contains the following underlying data:

- images (folder containing the set of images used for the data analysis)
- microscopy_measurments.csv (measurements acquired with the microscope software)
- Software_ThicknessAnalysis.jl (main script for reproducing the data analysis)
- Functions_ThicknessAnalysis.jl (collection of functions for the data analysis)
- Manifest.toml and Project.toml (computational environment files)
- _init_.jl (utility script for reproducing the computational environment)
- README.md

Extended data

Zenodo: Fabrication of Hydrogel Mini-Capsules as Carrier Systems - Extended data. https://doi.org/10.5281/zenodo.8413661[24].

This project contains the following extended data:

- ChargedCapsuleCaCO3.gif (time-lapse of an hydrogel capsule filled with CaCO3 to show the nature of the core through the release of the particles)
- ChargedCapsuleSiO2.jpg (image of an hydrogel capsule loaded with SiO2 microparticles)
- ChargedCapsuleSiO2_core.wmv (video of the particle in the picture - "ChargedCapsuleSiO2.jpg" after shell dissolution)
- TutorialBeadsFabrication.mov (video tutorial showing how the fabrication of the core-shell hydrogel capsules is realized)
- TimelapseCapsuleDyeReleased.png (release of a green dye from one capsule monitored over 30 minutes)
- CapsuleInsideSiliconeTube.mov (video of the capsule passing through the silicone tube)
- LowMeltAgaroseCapsule.png (images of a core-shell capsule fabricated using low-melting agarose)

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Open Peer Review

Current Peer Review Status: ✔ ✔ ? ? ✔

Version 3

Reviewer Report 22 August 2025

https://doi.org/10.21956/openreseurope.22698.r57058

✔ **Dr. Sudhir G. Warkar**
Delhi Technological University, Delhi, India

The authors have addressed the concerns I raised in my peer review report appropriately, and the paper can be accepted for indexing

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biopolymer-based Hydrogels for agricultural, drug delivery, water-enrichment and sensing applications.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 06 August 2025

https://doi.org/10.21956/openreseurope.22698.r57059

✔ **Min-Soo Kim**
ETH Zurich, Zurich, Switzerland

The authors have addressed my concerns.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of

expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 10 January 2025

https://doi.org/10.21956/openreseurope.20144.r48108

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Min-Soo Kim
[1] ETH Zurich, Zurich, Switzerland
[2] ETH Zurich, Zurich, Switzerland

This manuscript introduces a fabrication method for hydrogel-based core-shell microcapsules intended for drug delivery applications. A parametric study on shell thickness, influenced by the crosslinking agent ($CaCl_2$) concentration, revealed that the shell thickness varies between 100 μm and 800 μm with ~2 mm of core diameter. The proposed manufacturing technique offers an advantage as it does not require organic solvents, surfactants, or extreme pH conditions. The vision-based shell thickness measurement strategy also presents potential utility for microcapsule research. Below are concerns the authors should address:

1. Additional mechanical tests are necessary to demonstrate the deformability of the microcapsules, such as measuring deformation under applied pressure or force.
2. The lack of shell formation at lower $CaCl_2$ concentrations (threshold behavior) should be supported by additional experimental evidence to substantiate this claim.
3. In practical applications, how much drug can be loaded into the microcapsules as a percentage of their volume? What types of drugs can be incorporated into the core? How is the alginate shell dissolved at the targeted site, and what alternatives to sodium citrate dihydrate can be used for shell dissolution?
4. The authors should clarify the image-based thickness measurement method. It is essential to clarify whether the red-blue channel intensity is measured solely at the center point or across a region between the center and the background.
5. The fabrication steps for loading core materials (e.g., SiO_2 or $CaCO_3$) described in Section 2.3.2 should be moved to precede Section 2.3 for better logical flow.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microrobotics, Microfabrication, Micro mechancial test

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 14 Jul 2025

Elisa Roberti

1. See answer to Question 3 of Samuel Stealey. **2.** We cannot entirely rule out the presence of an alginate shell – we do not claim the lack of shell formation, although we suspect this is indeed the case. Nonetheless, even if present, the shell appears to be extremely thinner than anticipated (and perhaps discontinuous) and definitely not useful for the purpose of our research. **3.** In this preliminary work, we did not envision any specific drug molecule or drug loading method. Nonetheless, we expect to be able to load substantial amounts of drug-loaded nanoparticles (realistically few tens % in volume). The dissolution of the microrobots in SCD was performed solely to assess the consistency of the core. The shell is extremely permeable to small molecules (we verified it with a simple dye – see *Extended data*) and sufficiently permeable even to larger molecules, thus eliminating the need for its dissolution under physiological conditions. **4.** The red-blue channel intensity is measured in a central area to avoid edge effects. Before doing that, the script clusters the pixel of the images into capsule and background. The central region of each capsule is chosen for the color-based analysis as it is sufficiently homogeneous and corresponds to a portion of the shell that is approximately on the plane perpendicular to the light path (from illumination to objective). The analysis code is also provided with the report's underlying data and can be reproduced following the provided instructions. **5.** The step mentioned by the reviewer is not part of the devised procedure for fabricating the core-shell capsules, and for this reason, it was not included in Section 2.3. The addition of SiO_2 or $CaCO_3$ was inserted as a modification of the procedure to perform subsequent tests to verify whether the core was indeed in the liquid state

Competing Interests: No competing interests were disclosed.

Reviewer Report 05 December 2024

https://doi.org/10.21956/openreseurope.20144.r46291

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Bradley Nelson
[1] Multi-Scale Robotics Lab, Zurich, Switzerland
[2] Multi-Scale Robotics Lab, Zurich, Switzerland
Fabian Landers
[1] D-MAVT, ETH Zurich, Zürich, Zurich, Switzerland
[2] D-MAVT, ETH Zurich, Zürich, Zurich, Switzerland

The manuscript introduces a fabrication method for hydrogel-based core-shell capsules designed for drug delivery. The authors present an intriguing approach to achieving a liquid core without relying on extreme pH conditions or organic solvents, which is advantageous for sensitive drugs. Using different cross-linking mechanisms is an elegant solution for creating core-shell structures. Additionally, introducing a shell thickness measurement protocol is a valuable contribution that could benefit other researchers in the field.

The authors should address the following comments:

1. The authors highlight the benefits of "topical" drug administration at several points. Is the topical route going to be the primary route of delivery? If not, the term "local" may be more suited in this context.
2. In the first part of the introduction, the authors describe the advantages of local drug delivery. While these statements are valid, adding more references would better illustrate the scope and potential of solving this challenge.
3. The authors state that a double-emulsion process is unsuitable for fabricating ultra-deformable microrobots. Clarification is needed here: why exactly is this process not suitable?
4. It is unclear which specific drug the authors envision integrating into their microrobots. While the fabrication process avoids extreme pH conditions and organic solvents, it requires high temperatures (90–95 °C for 2–7 hours) to melt the core. What types of drugs would be compatible with these conditions? Small-molecule drugs could be a good candidate, but would diffusion out of the microrobot pose a problem? The authors mention avoiding dyes for shell staining due to diffusion challenges, which raises concerns about potential drug leakage.
5. The proposed protocol for measuring shell thickness is an interesting and innovative method that addresses a critical issue. However, its validity should be confirmed using an established method for comparison.
6. The dissolution of the microrobots is achieved by immersing them in the SCD solution. How could such conditions be replicated in vivo?
7. An analysis of the polydispersity of the microrobots should be added to provide insight into the fabrication process.
8. Mechanical testing of the microrobots' deformability is necessary to substantiate the claim

of ultra-deformability. Simple tests like flushing the microrobots through various pore sizes would provide supporting evidence.

9. Finally, the potential impact of nanoparticles on the formation of the alginate shell is not discussed. This interaction should be evaluated to ensure it does not interfere with the shell formation process.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Micro/nano robotics

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 14 Jul 2025

Elisa Roberti

1. We thank the reviewer for the clarification; we have revised the main text accordingly. **2.** The introduction section was revised according to the reviewer's suggestions. **3.** Core-shell particles are commonly produced using double-emulsion methods, which indeed could be suitable for obtaining ultra-deformable microrobots. However, emulsion methods rely on having two immiscible phases, typically an aqueous phase and an oil phase. For this reason, to obtain an aqueous core, emulsion methods usually imply the usage of oil-soluble, hydrophobic polymers as the main constituent of the shell. To allow their content to be released, emulsion-made core-shell capsules usually require the impermeable shell to break or degrade. In our case we aimed at a different approach, which would not require compromising the structural integrity of the microrobot for release. We thus decided to explore the possibility to fabricate core-shell microrobots with both an aqueous core and

the shell consisting of an aqueous material (hydrogel), which however could not be achieved by double-emulsion methods. **4.** In this preliminary work, we did not envision any specific drug molecule as we aimed at defining a process to make all-aqueous core-shell microrobots. The polymer used for the core template (in our case agarose) can be changed (e.g. using low melting temperature agarose or gelatine, see *Extended Data*) to adapt this process to specific drug carrying applications. Concerning the uncontrolled diffusion of the drug, we foresee our microrobots as carriers of drug-loaded nanoparticles rather than of free drug molecules. As for the iron oxide nanoparticles we have used to stain the shell, we expect drug-loaded nanoparticles to not diffuse out of our microrobots uncontrollably. The release could then occur either from the particles immobilized inside the microrobots' constituent gel, or by the stimuli-triggered release of drug-loaded nanoparticles (alginate gels such as those used for the shell undergo substantial swelling/deswelling in response to, e.g. pH changes). Section 4 is revised accordingly. **5.** The color-based method for correlating the shell thickness to the color intensity directly relies on the fitting of Equation 3 to actual, microscopy-measured thicknesses (see comparison made in Figure 4b). Outside of the interval of actually measured thicknesses, simple extrapolation allows for comparison of expected (from the expected thickness) and observed color intensity. Being dependent on the fitting on actual data, this method only allows for interpolation or extrapolation close to the fitting interval. We believe that further validation and generalization is beyond the scope of this brief report. **6.** The dissolution of the microrobots' shell in SCD was evaluated solely to confirm that the core was indeed liquid and is not the intended in vivo release strategy. As for the release method, our microrobots are envisioned to rely on the responsiveness of encapsulated drug-loaded nanoparticles. Once the drug is released by the nanoparticles, it will steadily diffuse through the permeable shell. Alternatively, the release can be obtained by a change in the swelling state of the hydrogel shell (see answer to Question 4). **7.** See answer to Question 2 of Samuel Stealey. **8.** See answer to Question 3 of Samuel Stealey. **9**. The nanoparticles used to enhance the visualization of the alginate shell are approximately three orders of magnitude smaller than the shell itself. Given their significantly smaller size and the low concentration employed (0.2 mg/mL), we do not anticipate any adverse impact on the shell formation process.

Competing Interests: No competing interests were disclosed.

Reviewer Report 28 November 2024

https://doi.org/10.21956/openreseurope.20144.r46297

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Samuel Stealey
[1] Saint Louis University, Saint Louis, USA
[2] Saint Louis University, Saint Louis, USA

The manuscript by Roberti et al. demonstrates an attractive option for forming microrobots using only mild conditions and non-organic solvents. Such a system is attractive for potential use for drug delivery applications. Here are some minor comments the authors should address to enhance the discussion of their results:

1) With the intended end application being drug delivery, can the authors describe how therapeutics may be loaded within the microbots? For example, would these therapeutics be added to hydrogel precursor solutions prior to core-shell formation? If so, could the loaded therapeutic diffuse out throughout and following the subsequent washing/melting/gelation steps? If therapeutics were to be loaded following stable, core-shell formation, have others in the literature achieve sufficient drug loading?

2) The authors should report the polydispersity in of the measured agarose cores within the manuscript. It is implied that they are quite monodisperse, but with the small sample sizes measured, it is difficult to judge the degree to which they are of similar size.

3) Could the authors employ rudimentary methods to assess deformability? While advanced and precise techniques such as nano-indentation and flow through capillaries may be outside the scope of this feasibility study, it may be beneficial to manually compress the relatively large spheres and observe the effect on microbot size and structure. For example, a glass slide could be added on top of the large spheres, allowing for imaging during and after deformation.

4) The authors should discuss the feasibility of reducing the size of the agarose core beads to physiologically relevant sizes. Can this be achieved using a similar method, or would another method be required?

5) Following the "melting" of the agarose core, can the authors speak to the relative molecular weight of the agarose polymers? Would these non-gelled polymer strands be expected to diffuse out of the aqueous interior, or would they remain in the core, potentially providing for a crowded environment that could slow diffusion marginally?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Hydrogel microspheres, biomaterials testing

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 14 Jul 2025

Elisa Roberti

1. In nanosystems, drug loading strategies are generally classified into three main categories: pre-loading, co-loading, and post-loading (Liu et al., 2020).

- pre-loading involves first producing drug nanoparticles and then surrounding them with a stabilizing and protective shell.
- co-loading refers to encapsulating the drug during the formation of the nanocarrier itself.
- post-loading is the process where nanocarriers are fabricated first, followed by the incorporation of the drug into the pre-formed carrier.

According to the literature, the most common approach involves mixing the drug with an alginate dispersion before gelation (co-loading), a method that does not interfere with the drug's stability (Rajapaksha et al., 2024). In our specific protocol, agarose is incorporated during the initial core formation phase and subsequently removed by heating the system to 90–95 °C. For applications involving drugs or molecules that are sensitive to high temperatures, it is worth noting that we successfully fabricated similar core-shell capsules using low-melt agarose as a template core, which has a melting temperature of approximately 60°C. In the post-loading strategy, the drug is encapsulated by exploiting the pH-responsive swelling and deswelling properties of alginate (Malektaj et al., 2023). By adjusting the pH, the alginate matrix can swell, allowing for drug loading after the thermal steps, and then deswell to entrap the drug, thus avoiding exposure to high temperatures. To mitigate potential leakage from the core-shell capsules, we envision a strategy that involve loading the drug not in its free form but conjugated to nanoparticles, which are in turn loaded within the capsules. This approach aligns with current practices in nanoparticle drug delivery, where drugs are conjugated to or encapsulated within nanoparticles to enhance stability and control release profiles. Responsive nanoparticles could thus be used to obtain targeted /triggered drug release, whereas the capsule carriers will allow for controlled transport and local administration. We have modified the introduction and the conclusion section accordingly. **2.** The PDI of the agarose cores is ~0.009, calculated as σ^2/μ^2 over the totality of cores (i.e. 38 samples). The value tells us the samples show good uniformity (PDI < 0.1), as can be seen also from the distribution histogram reporting radius vs frequency counts (see Underlying Data). **3.** We thank the reviewer for this comment. To preliminary assess the deformability of the core-shell capsules, we performed a simple deformability test, which is described in the new version of the manuscript (Section 2.3.3, Section 3.3). We would also like to note that a quantitative estimation of the deformability would indeed be important and could be the focus of a follow-up work. **4.** The size limitation

arises from the use of syringe needles in the initial formation of the cores. By employing a method capable of producing smaller droplets and, thus, smaller cores (e.g. microfluidics or spray), this approach could potentially be adapted for the fabrication of smaller capsules. We have briefly commented on this aspect in the Conclusions. **5.** The relative molecular weight of agarose polymers typically ranges from 120,000 to 150,000 Daltons (Da). Ideally, agarose should melt and diffuse out of the alginate shell at 90°C, given the high permeability of alginate. Although we observed that some gel traces remain within the aqueous core, the most part of it actually leaves the core as we can see from the changes of the core structure (for more details, see Extended Data). While this may affect the internal diffusion marginally, the limiting factor will be the diffusion through the shell. Bibliography Liu, Y., Yang, G., Jin, S., Xu, L., Zhao, C.-X., 2020. Development of High-Drug-Loading Nanoparticles. Chempluschem 85, 2143–2157. https://doi.org/10.1002/cplu.202000496 Malektaj, H., Drozdov, A.D., deClaville Christiansen, J., 2023. Swelling of Homogeneous Alginate Gels with Multi-Stimuli Sensitivity. International Journal of Molecular Sciences 24, 5064. https://doi.org/10.3390/ijms24065064 Rajapaksha, W., Nicholas, I.H.W., Thoradeniya, T., Karunaratne, D.N., Karunaratne, V., 2024. Novel alginate nanoparticles for the simultaneous delivery of iron and folate: a potential nano-drug delivery system for anaemic patients. RSC Pharm. 1, 259–271. https://doi.org/10.1039/D3PM00068K

Competing Interests: No competing interests were disclosed.

Reviewer Report 06 November 2024

https://doi.org/10.21956/openreseurope.20144.r45505

Mahmut Selman Sakar
[1] Ecole Polytechnique Federale de Lausanne, Lausanne, Vaud, Switzerland
[2] Ecole Polytechnique Federale de Lausanne, Lausanne, Vaud, Switzerland

The revisions are appropriate. The manuscript can be accepted without further changes.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microrobotics, microfabrication, hydrogels, magnetic actuation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 22 February 2024

https://doi.org/10.21956/openreseurope.18062.r37273

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Dr. Sudhir G. Warkar
[1] Delhi Technological University, Delhi, India
[2] Delhi Technological University, Delhi, India

Comments of Reviewer:
In the paper entitled “Fabrication of hydrogel mini-capsules as carrier systems” the authors proposed the synthesis of microrobots made as deformable capsules with hydrogel shells and aqueous cores, having the potential added advantages of biocompatibility, permeability, and stimulus-responsiveness. The process of forming alginate hydrogel mini capsules proceed through the dripping of the mixture of alginate and agarose into a calcium chloride solution to form crosslinked shell of alginate and agarose core. The cross-linked alginate shell is de-crosslinked by dipping the beads in sodium citrate dihydrate solution.
The scientific quality of the paper is good and the scientific study of the paper is relevant to the academic and scientific community. The paper also has adequate references cited. However, the manuscript needs a few minor changes/clarification on the following observations:

Reviewer has observed the following issues with the manuscript which the authors must address:

1. The manuscript requires more clarity for better understanding and repeatability.

2. Section 2.2, after de-crosslinking the alginate template, agarose beads were left behind which were transferred to $CaCl_2$ solution of different concentrations to form shells of different thicknesses. Authors should clarify in the paper whether these shells formed on agarose beads are of which material.

3. If both agarose and alginate get crosslinked by Ca^{2+} then why does only alginate get de-crosslinked on immersing in sodium citrate dihydrate.

4. Section 2.2, a more detailed description of step 5 is required- such as before placing the beads at a temperature of 90-95^{o}C, agarose was in which physical state (solid/liquid). What exactly happens at 90-95^{o}C? [In the abstract, authors mentioned ‘Finally, the beads are heated to let the agarose melt and diffuse out, leaving a liquid core’.]

5. More explanation is required for the loading and release of $CaCO_3$ and SiO_2 in the core.

6. The shell thickness of capsules was measured from microscopy images by measuring the colour intensity-whether the results can be validated by other characterization techniques?

7. The insights need to be incorporated in the paper related to how the hydrogel mini capsules

developed as biomedical microrobots can be used for controlled navigation, which could allow them to be guided to hard-to-reach target sites inside the human body.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Polymeric hydrogels for drug delivery, agriculture, metal ion sensing, and water enrichment applications.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 24 Sep 2024

Elisa Roberti

1. Thank you for your feedback. Please consider that in addition to the manuscript, we have uploaded a video demonstrating the protocol. We believe that this provides a clearer visualization and a better understanding, and that it enhances repeatability. In addition, we have addressed all reviewers' comments to further making our manuscript and process clear, easy to understand and reproduce.
2. The agarose beads are submerged in calcium chloride solutions of varying concentrations to absorb the solution. When the CaCl2-loaded agarose beads are transferred into a fresh alginate solution (containing iron oxide nanoparticles), the calcium ions diffuse out, immediately crosslinking the alginate around the agarose beads, forming the shell. The shell is thus made of a Ca-alginate hydrogel containing iron oxide nanoparticles.
3. It is worth clarifying that only alginate crosslinks due to the presence of calcium ions, while agarose forms a gel via a physical mechanism when cooled from high

temperature to room temperature. We indeed use these different hydrogels, exploiting their distinct gelation mechanisms, exactly to be able to de-crosslink them selectively.

4. Agarose remains in a gel/solid phase at room temperature and liquefies at temperatures above 90°C. To enable the liquefication of the agarose gel and the diffusion of agarose molecules through the alginate shell, we heat the core-shell particles (with agarose as the core and alginate as the shell) to 90-95°C, ideally resulting in an agarose-free liquid core surrounded by an alginate shell. However, we have observed that agarose does not fully diffuse out as expected.
5. In microrobots fabrication, it can be useful to load not only magnetic nanoparticles, but also microparticles of other kinds. As an example, we tried these two different kinds of particles, with $CaCO_3$ being also sensitive to acidic pH. SiO_2 microparticles were loaded in the agarose solution before realizing the cores, while $CaCO_3$ microparticles were nucleated directly in the already-made cores as an alternative loading method, through the reaction of $CaCl_2$ with Na_2CO_3. The aim of this experiment was to verify the possibility to load or nucleate particles inside the core, as well as to aid the visualization of the final consistency of the core: we thus dissolved the shell and observed how the loaded particles were released to infer whether the core was fully liquid or had the consistency of a very soft gel.
6. The shell thickness was also measured directly with the measuring tool of our digital microscope, for the capsules whose shell was thick enough to be detected. The colour intensity calculation was performed to also extrapolate data for shells that were not directly measurable by optical microscopy. Our results suggest that there is in fact no substantial/continuous shell for low $CaCl_2$ concentrations.
7. *The "Conclusions" section was revised to address the question.*

Competing Interests: No competing interests were disclosed.

Reviewer Report 23 January 2024

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Mahmut Selman Sakar
[1] Ecole Polytechnique Federale de Lausanne, Lausanne, Vaud, Switzerland
[2] Ecole Polytechnique Federale de Lausanne, Lausanne, Vaud, Switzerland

This article introduces a technique to fabricate capsules with an alginate shell and liquid core. The technique is described in detail, accompanied by a video that contains step-by-step descriptions. The shell thickness is measured using optical images. Two methods to estimate the shell thickness are proposed: from the $CaCl_2$ concentration and from the shell colour intensity. The colour

difference is due to the presence of iron oxide nanoparticles. Here are some comments on the rationale behind the design of the capsules and the presentation of the methodology.

1. Authors hypothesize that the ability to move across tissues and barriers requires "ultradeformability". Please provide a technical description of "ultradeformability". How do we measure the deformability of a liquid-filled soft shell? How does this measure depend on shell thickness, mechanical properties of the shell, and the viscosity of the liquid in the core? What is the role of osmotic pressure?

2. What is the desired level of deformability? Where is this specification come from (i.e., the size of the holes in the barrier)? Do we know how deformable the immune cells are as a starting point? Can they penetrate into solid tumour? I brought up solid tumour as this is the case mentioned by the authors in the introduction. If the immune cells cannot, would it be reasonable to believe that an engineered capsule can?

3. As a follow up to the first two comments, what is the liquid inside the capsule? If the liquid inside the capsule is molten, de-polymerized agarose instead of water as intended, would that influence the deformability of the capsule or the release of the cargo?

4. Please substantiate the claim that none of the existing fabrication methods are suited to fabricate core-shell capsules without damaging potential payloads. There are numerous articles on the fabrication of hydrogel-containing core–shell capsules, specifically alginate or chitosan as the shell material. Majority of these articles show encapsulation and release of drugs. What am I missing here?

5. The alginate capsule is permeable and highly deformable. How would the cargo be protected while crossing barriers? How do you envision to implement selective permeability in order to protect the cargo and release at the target location? Is this strategy compatible with the way the capsules are fabricated?

6. I could not find statistics on how many capsules were fabricated, size range, and dispersity. Why do we see few data points on the plots? Considering the throughput of the fabrication method, I would expect to see many measurements per condition (e.g., $CaCl_2$ concentration) with mean and standard deviation instead of a single data point.

7. Why is the volume of the alginate shell V_s proportional to the amount of Ca^{2+} ions in the core. Please plot shell thickness with respect to capsule size. That would nicely accompany your illustration shown in Figure 2.

8. What is the effect of adding iron oxide nanoparticles into the alginate to the stiffness of the shell? Is this strategy compatible with the overall objective of making "ultradeformable" capsules?

9. The discussion does not address an important issue. What future steps will transform the core-shell capsules described in this work into microrobots? How do you envision to implement the required fabrication steps and control strategies? What are the trade-offs and challenges in terms of material choice and design parameters? For example, if magnetic force is going to be applied to pull these capsules through barriers, magnetic volume must be increased which will inherently constrain the deformability of the shell.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microrobotics, microfabrication, hydrogels, magnetic actuation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 24 Sep 2024

Elisa Roberti

1. We thank the reviewer for this question, which allows us to better explain the concept of ultra-deformability in the context of micro robotics. In the literature, the term "ultradeformability" is primarily associated with liposomes or transferosomes, particularly those used for transdermal delivery. In that specific scenario, there is a requirement for vesicles capable of substantial squeezing to pass through skin pores without losing their cargo (Souto et al., 2021; Sharma et al., 2024). We broadly define “ultra-deformability” as the ability of microrobots/structures to deform substantially under small external forces (same order of magnitude as those needed to drag them through a liquid) and to pass through openings that are smaller than their size (Vorselen et al., 2020)(Simrah et al., 2024). Possible methods to measure deformability could involve compelling these soft beads to traverse a capillary, nanoindentation, or AFM in force distance curve-based imaging mode. (Vorselen et al., 2020). These techniques are also commonly employed for liposomes or polymersomes. Lowering the thickness and/or elastic modulus of the shell reduces its bending and tensional stiffness, thus increasing the deformability/compliance of the whole structure. The viscosity of the liquid core influences the rate of deformation, as

well as the effective deformability of the structure (the lower the viscosity, the lower the forces/stresses required for the liquid to flow within the core). The viscosity can play an additional role if the mechanism of deformation involves a change of volume and flow of the core liquid through the porous shell (the lower the viscosity, the lower the flow resistance). As for the osmotic pressure, its changes can induce swelling or deswelling of the shell and structure, leading to a volume change and change of mechanical properties of the shell. We have thus revised our manuscript to provide an explanation of the concept of ultra-deformability.

2. Leukocytes are sufficiently deformable and elastic to move inside the small capillaries (diameter < 5 μm) that reach solid tumors, to travel through the interstitial spaces of tissues (< 1 μm), and to extravasate through the nanometric junctions between the endothelial cells of the leaky vasculature in the tumor microenvironment (100 to 500 nm (Wilhelm et al., 2016), depending on the tumor type and stage). Leukocytes can indeed migrate to and infiltrate inside tumors (Wu et al., 2022). If deformable enough, capsules of diameter $\gtrsim$ 10 μm should be able to at least move in small capillaries. By making them smaller or even more deformable, we could potentially enable their extravasation from blood vessels, particularly considering the gaps between endothelial cells in tumor tissue. Nonetheless, here we report a preliminary investigation of a fabrication method potentially useful for preparing ultra-deformable capsules. The assessment of the fabrication method is performed on millimeter-sized capsules to ease the process and analysis, and further effort would be needed to adapt the method to make microcapsules of the size required for the envisioned applications.
3. Our initial assumption was that once the agarose melted, it would easily diffuse out of the shell, leaving a completely liquid aqueous core. However, we realized that although the agarose melts, the core is not fully liquid (see Extended Data). This could negatively affect the deformability of the capsules. Nonetheless, we have chosen the lowest concentration of agarose that allows the formation of a gel in the initial steps of the process, to minimize the amount of residual agarose in the core after its melting and diffusion out of the shell. With this in mind, we assume that the non-complete liquefication of the core will eventually affect the deformability of the capsule, yet only negligibly. For what concerns the release of the cargo, cargo diffusivity, hydrophilicity and dimensions will have to be taken into account, but we do not expect agarose residues inside the core to make a substantial difference, as deformability and cargo release will mostly depend on the shell properties.
4. We agree that many processes exist to fabricate hydrogel-containing core-shell capsules, e.g. the template-based formation of alginate-chitosan capsules through the layer-by-layer method. However, we have been looking for methods that lead to capsules where both the shell and the core are aqueous, that allow the core to be liquid, and that use only very mild conditions (e.g. ~neutral pH in all steps, to allow the loading of pH-sensitive cargos). None of the reported fabrication methods, to the best of our knowledge, have all the features we need for our purposes (e.g. LbL deposition of chitosan requires acidic pH). Therefore, we have conceived this new fabrication process, and we report here a preliminary assessment of the produced capsules, confident that it could complement the various fabrication processes already reported in the literature.
5. Alginate nano and microparticles have been extensively used to obtain optimal drug

delivery systems (Hariyadi and Islam, 2020; He et al., 2020; Abourehab et al., 2022), due to some of the properties of this hydrogel such as the low toxicity, chemical versatility, and pH sensitivity. Notably, there are many examples of alginate-based drug delivery systems capable of targeting and releasing cargos under specific conditions, such as at the low acidic environment near tumors (He et al., 2020). Ideal drug-delivery systems are expected to securely encapsulate drugs without leakage, before reaching the target site and release the drugs upon reaching the target. Alginate-based nanoparticles can accumulate at the target site via passive or active targeting, but some strategies are needed to disassemble the capsule or increase permeability, developing stimuli-responsive capsules. In our specific case, the deformability for crossing the barriers is firstly given by the bending of the shell, and in part by its extension/compression, aiming at minimizing the inner volume changes so as to minimize the exchange of liquids between the core and the environment, decreasing the cargo losses while crossing barriers. Moreover, the volume change can be stimuli-induced and used to trigger the cargo release.

6. We realized many samples, then we thoroughly analyzed from 3 to 5 particles per each experimental condition, and considering the high reproducibility of the results, we assumed it to be sufficient. Then considering the samples number, we thought it was more meaningful to
report the values of the single samples rather than mean and standard deviation.
7. *The thickness, and thus the volume of the alginate shell is proportional to the amount of* Ca^{2+} *ions that were previously loaded into the core because it determines the number of cross-link bonding that each bead can realize. Once the core is put in a solution at a fixed alginate concentration, the* Ca^{2+} *ions will be released, inducing the crosslinking of the alginate in the nearby of the cores, and producing a shell whose thickness will be (at first approximation) determined by the number of* Ca^{2+} *ions loaded in the core itself (which could be approximated as the product of* $CaCl_2$ *concentration times the volume of the core, dealing with a hydrogel which is mostly* water). Nonetheless, the most important parameter that we are changing here is the $CaCl_2$ concentration, whereas there is no large variation among the core radii, with the small polydispersity observed being due to the fabrication method. Moreover, the simple reported model assumes that the core has constant volume during the process, whereas we observed experimentally that the cores undergo a certain degree of swelling and deswelling during the process, also depending on the concentration of $CaCl_2$. For these reasons, we have not included the resulting plot of shell thickness versus capsule core radius as it is not informative and shows no clear correlation in the small range of radii observed.
8. The current amount of nanoparticles used is very low, sufficient only for a clear visualization of the capsules but irrelevant from a deformation standpoint. To obtain magnetic microrobots, much higher concentrations of magnetic nanoparticles (or ferrofluid) will be needed. However, by using nanoparticles with a diameter of less than 10 nm and potentially placing them in the core instead of the shell, the impact on deformability will be minimized.
9. *The "Conclusions" section was revised to address the question*.

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Competing Interests: No competing interests were disclosed.