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Ashkan Madadlou (Conceptualization) (Writing - original draft), Vittorio Saggiomo (Writing - original draft), Karin Schroën (Writing - review and editing), Vincenzo Fogliano (Writing - review and editing)

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All-aqueous emulsions as miniaturized chemical reactors in the food and bioprocess technology

Ashkan Madadlou¹, Vittorio Saggiomo², Karin Schroën³,⁴, Vincenzo Fogliano¹*

¹Food Quality and Design Group, Department of Agrotechnology and Food Sciences, Wageningen University and Research, Wageningen, P.O. Box 17, 6700 AA Wageningen, The Netherlands

²Laboratory of BioNanoTechnology, Wageningen University and Research, P.O. Box 8038, 6700, EK Wageningen, The Netherlands

³Food Process Engineering Group, Department of Agrotechnology and Food Sciences, Wageningen University and Research, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands

⁴Membrane Processes for Food, University of Twente, Drienerlolaan 5, 7522 NB Enschede, The Netherlands

Highlights

- Aqueous two-phase systems can be miniaturized to all-aqueous emulsions
- They can be used to accomplish numerous reactions within droplets and on droplets.
- In-droplet reactions include extractive bioconversion, and microgel synthesis
- On-droplet reactions include microcapsule formation and interfacial catalysis
- Microfluidics enables control over formation and exploitation of the emulsions

Abstract

All-aqueous emulsions are conventionally formed at bulk scale by mild shaking of aqueous two-phase systems. They can be used to carry out reactions both in droplets (compartmentalized) and on droplet surfaces in conditions free of synthetic surfactants and organic solvents. The use of all-aqueous emulsions for extractive bioconversion is a routine application; however, these emulsions hold many more promises. A renowned,
rapidly evolving application is bio-microgel synthesis through biopolymer crosslinking within the emulsion internal phase. When polyelectrolyte crosslinking is achieved at the interface rather than in droplets, microcapsules can be formed, and when in situ colloidal particle generation at the droplet surface is obtained, colloidosomes are produced. The use of microfluidics to control the formation of all-aqueous emulsions offers many advantages in reactions monitoring and partitioning of reactants.

*Keywords*: Emulsion; All-aqueous emulsion; Reaction; Microreactor; Food Reaction

**Conventional emulsion microreactors**

Miniaturized chemical reactors provide inimitable advantages over running reactions at large scales, including reduced use of chemicals and solvents, increased safety, enhanced reaction rates, and the ability to screen and vary reaction conditions pretty much at will [1]. Various sorts of microreactors such as liquid marbles and liquid biphasic systems (LBSs, liquid-liquid systems) have been developed and exploited. Oil-water LBSs can be effectively miniaturized by standard emulsification (bulk scale), and within fluidic microchannels which allows unprecedented control over droplet size. Running reactions in oil-water LBS microreactors enables combining immiscible partners of a reaction at the oil-water interface [2,3]. Alternatively, the interface might serve as the purging site for removal of specific compounds produced in either of the emulsion phases into the opposite phase. For example, oil-in-water emulsion droplets stabilized by lipase-loaded mesoporous carbon nanospheres functioned as a microreactor with reactants in the oil phase for enzymatic transesterification of phytosterols and concurrent removal of the produced H₂O across the oil-water interface into the aqueous phase [4]. Likewise, the
aqueous phase of water-in-oil emulsions can be used as compartmentalized microreactors, for instance, for synthesis of protein [5], peptide [6], and starch [7] microgel particles by enzymatic, chemical and thermal crosslinking. Although in limited cases e.g. during hydrolysis of vegetable oils, surface active products can be formed, synthetic surfactants are very commonly required to decrease the interfacial tension at the oil-water interface, and thus facilitate emulsification. However, these surfactants cause high carbon footprints during their production, and are detrimental to human health and the environment. To mitigate this, W/O Pickering emulsions that are free of surfactants [2] have been used; however Pickering particles are not necessarily intrinsically safe, and they may be cumbersome to prepare. Besides, tedious purification steps that involve the use of organic solvents that subsequently have to be fully removed are needed. Moreover, oil oxidation may occur during production, potentially jeopardizing quality and safety of the final product.

All-aqueous emulsions: a green alternative

All-aqueous emulsions are genuine alternatives to water-oil emulsions and can be used for chemical reactions without the need of synthetic surfactants and organic phase. These emulsions are aqueous two-phase systems (ATPSs) and comprise of two immiscible aqueous solutions. The interfacial tension between the immiscible aqueous phases is several orders of magnitude lower than at typical oil-water interfaces [8]. This allows formation of microdroplets by mild mechanical forces, i.e. shaking/stirring at bulk scale and perturbation of water-water (W-W) jets in microfluidic channels, yielding all-aqueous emulsions [9]. The thickness of the W-W interface typically exceeds the effective length of the constituent polymers and the ultralow interfacial tension basically takes away the driving force for low molecular weight surfactants to adsorb at the interface. Comparable to the oil-water interface, particles can accumulate at the W-W
interface, which hinders spinodal decomposition and macroscopic phase separation of all-aqueous emulsions [10].

Components that can form all-aqueous emulsions are pairs of i) noncharged polymers e.g. polyethylene glycol (PEG)-dextran (Dex), ii) a noncharged polymer (e.g. PEG) with a salt (e.g. K$_2$HPO$_4$) or with a charged polymer (e.g. chitosan), or iii) two co-charged polymers [11]. Dex is listed generally recognized as safe (GRAS) and PEG is a biocompatible polymer approved for human oral applications [12]. Within the food sector good quality ATPS were obtained using whey proteins which were hydrophobized through a food-grade acetylation procedure and heat treatment [13], resulting in immiscibility of the negatively charged proteins with Na-alginate [14]. Based on interfacial rheology assessments, it was found that the most hydrophobic particles accumulated at the W-W interface [15], and other particles remained in the protein phase.

**Partitioning in all-aqueous emulsions**

All-aqueous emulsions segregate guest molecules (such as DNA, proteins and enzymes) through partitioning between the two aqueous phases, as well as at the interface. Efficient partitioning of reactants between the two (inner and exterior) phases of emulsions is a prerequisite for accomplishing chemical reactions either in the inner phase or at the W-W interface. Partitioning in the inner phase enables running compartmentalized reactions, whereas partitioning at the interface allows to carry out on-droplet reactions. Within the most common pair, i.e. PEG-Dex, PEG forms the less polar phase [11] and it can be removed by gel permeation chromatography [16] once the reaction is accomplished. The utilization of thermo-separating polymers such as UCON (a copolymer of ethylene oxide and propylene oxide) facilitates polymer recovery
by heating above its lower critical solution temperature (LCST) [17]. Comparably, certain food-grade polymers, e.g. hydroxypropyl cellulose have an LCST of 43\(^\circ\)C at 0 M NaCl [18].

A plethora of enzyme and other biomolecules have been segregated/compartimentalized using ATPSs. In Table 1, examples of ATPSs with the segregated compounds are provided. Recovery of proteolytic enzymes from microbial fermentations into the PEG-rich phases of PEG/phosphate and PEG/Citrate is also known [19]. The protease partitioning efficacy is dependent on PEG molecular weight, pH, and NaCl concentration [20]. PEG retains a higher enzymatic activity of the recovered recombinant chymosin, compared to other affinity phases e.g. poly(ethylene glycol)-block-poly-(propylene glycol)-block-poly-(ethylene glycol) [21].

**Compartmentalized reactions**

*Extractive bioconversion using all-aqueous emulsion reactors*

All-aqueous emulsions are widely used for extractive bioconversion. To this end, a pair of phase-forming polymers (without being stabilized by Pickering particles) are mildly stirred typically for hours to days, and consecutively separated [22]. Extractive bioconversion relies on preferential partitioning of the biocatalyst (microbial cells, enzymes) and its substrate in one of the aqueous phases (usually droplets) and recovery of the bioconversion product(s) into the other phase. Product partitioning away from the biocatalyst can e.g. lift product inhibition, and drive the reaction to higher conversion rates, as well as prevent cell toxicity [11,23].

Product partitioning into the recovering phase can be improved by adding specific ligands coupled to a phase-forming polymer and engineering of the product, for example via tagging the recombinant proteins [22]. Fermentative production of pullulan in PEG-
salt and PEG-Dex ATPSs and recovery of pullulan into the PEG phase [24] is a recent illustrative example of what all-aqueous emulsions can deliver to extractive bioconversion.

In the food sector, ATPSs have been used for the post-hydrolysis fractionation of food proteins. Recovery of bioactive peptides having inhibitory effects on angiotensin-converting enzyme from tryptic hydrolysates of casein was a pioneering case study; the peptides had a substantially greater affinity (yield ≥ 99%) towards the polymer-rich phase of PEG/potassium phosphate ATPSs [25]. As a recent instance, recovery of antioxidant peptides from hydrolysates of whey proteins was achieved using ATPSs of poly(ethylene glycol-ran-propylene glycol) monobutyl ether/phosphate [26] and 1-propanol/NaH₂PO₄ [27].

We expect that ATPS emulsions can be additionally exploited for the in situ generation of bioactive peptides from food proteins and concurrent fractionation of the peptides within either of the aqueous phases. This application would rely on preferential partitioning of food proteins and proteolytic enzymes within the inner compartment and extraction of the released peptides into the exterior phase. Since the phase behavior can be tuned extensively, we expect that several possibilities for industrial use of food-grade all-aqueous emulsions for proteolysis reactions are within reach.

**Fabrication of microgels using all-aqueous emulsions**

The utilization of all-aqueous emulsions for bioparticle (microgel) synthesis via solidification of the inner phase is appealing from the perspectives of consumer and environmental concerns. Synthesis of hydrogel microparticles through crosslinking of methacrylated dextran (mDex) in mDex-in-PEG or dimethacrylated PEG (m₂PEG) in m₂PEG-in-Dex (or m₂PEG-in-magnesium sulfate) emulsions were pioneering instances.
Similarly, gelatin microparticles were made by emulsification of a gelatin solution in an aqueous solution of poly(vinylpyrrolidone) or dextran at high temperature [28] and subsequent cooling to ambient conditions. In addition to solidification of the polymer which constitutes the internal phase, all-aqueous emulsions have been used as scaffolds for bioparticle synthesis via crosslinking of a guest ingredient in the dispersed phase. Recently, alginate which mainly partitioned into the inner phase of Dex-in-PEG emulsions was crosslinked with CaCl$_2$ to yield pollen-like microparticles (i.e. non-spherical vehicles with protruding spikes). For this, a flow-focusing microfluidic device (Figure 1) was employed to make alginate-Dex droplets (50-80 μm) in the PEG phase, followed by polymerization in a bath containing PEG and CaCl$_2$. In the bath, a sudden change in concentration across the Dex-PEG interface occurs, resulting in formation of alginate spikes across the droplet surface, of which the length could be tuned by the PEG concentration [29].

Comparable to ionic microgelation, which takes place between oppositely charged ions and biopolymers, electrostatic attraction between two oppositely charged biopolymers can be exploited for synthesis of microgel particles. Bicomponent protein microparticles consisting of hemoglobin (positively charged) and bovine serum albumin (BSA, negatively charged) or immunoglobulin G (negatively charged) were prepared in the droplet phase of Dex-in-PEG emulsions at pH 7.0 (Figure 2A). Before emulsification, the anionic and cationic proteins were placed in the Dex and PEG phases, respectively. Once the emulsion droplets were fabricated via a glass capillary-based microfluidic electrospray strategy, BSA remained in the Dex phase (partitioning coefficient was 0.96 in Dex and 0.04 in PEG), whereas, hemoglobin migrated from the PEG phase into the Dex phase (partitioning coefficient in Dex was 0.7). Gradual changes took place with the Dex droplets becoming darker because of the electrostatic attractive, as well as
hydrophobic interactions between the proteins [30]. Comparable methods can be used for preparation of bicomponent food protein, and protein-polysaccharide (positively charged proteins and negatively charged polysaccharides) composite microgels. Moreover, bio-microgels consisting of a charged bioactive cargo of interest can be encapsulated. The PEG phase needs to be removed for harvesting the bioparticles.

**Interfacial reactions using all-aqueous emulsions**

**Capsule formation**

Besides microgel formation, electrostatic attraction between charged species can be used for solidification of the interface, resulting in formation of microcapsules (i.e. the internal phase remains liquid). The first report [31]* included the self-assembly of oppositely charged polyelectrolytes, namely poly(allylamine hydrochloride) (PAH) and poly(sodium-4-styrenesulfonate) (PSS) at the W-W interface of all-aqueous PEG-Dex emulsions (Figure 2B). Formation of microcapsules (i.e. on-droplet self-assembly) rather than microgels (i.e. in-droplet self-assembly) requires several customizations. A key point is prevention of free diffusion of polyelectrolytes across the interface [31]*. As the interfacial tension in ATPSs is very low, polyelectrolytes are not adsorbed to the interface, but rather tend to migrate across the interface due to concentration differences between the phases. It is crucial to tune the relative fluxes of polyelectrolytes so that they meet at the interface. Moreover, the concentration of polymers in each aqueous phase has to be close to the equilibrium condition in the ternary phase diagram of the corresponding ATPS, so that, intermixing of the polymers is weak when the two solutions come into contact [32]. In order to tailor polyelectrolyte diffusion, they were added to the aqueous phase with poorer affinity. The polyanion PSS with much higher affinity for Dex (partitioning coefficient 0.69) was added to the PEG phase and the polycation PAH with almost no preference between the phases (partitioning coefficient
in Dex: 0.51) was added into the Dex phase. Once the Dex phase was electrospayed into the PEG phase, PSS strongly migrated from the continuous PEG phase inwards to the droplet phase (Dex), while PAH weakly migrated outwards. The opposite transfer of PSS and PAH caused complexation at the W-W interface, thus forming microcapsules [31].

Recently, BSA- or catalase-loaded colloidosomes were fabricated by in situ generation of colloidal particles at all-aqueous interfaces. Colloidosomes are microcapsules with shells consisting of colloidal particles. The dispersed phase, which consisted of Dex containing Na-alginate and urease (also BSA or catalase) was periodically injected into a continuous phase of PEG using a glass capillary microfluidic device (Figure 2C). Upon existing the microfluidic device, the emulsion droplets were directed into a bath containing PEG, CaCl$_2$ and urea: CaCl$_2$ and urea could freely diffuse across the interface and enter into the Dex phase, while, urease tended to stay in the Dex phase. The diffusion of Ca$^{2+}$ crosslinked the alginate at the droplet surface, and urease concurrently formed ammonium carbonate from urea that in the presence of Ca$^{2+}$ ions generated CaCO$_3$ particles. The CaCO$_3$ particles nucleated and grew into a solid shell on the microgels (Figure 2C). The encapsulation efficiency was high (>85%) [33].

Reactions at the droplet interface

The W-W interface can be exploited as a liquid-staying reaction site for reactants partitioned in the discrete phases of all-aqueous emulsions. To illustrate this, an enzyme-assisted oxidation reaction was investigated in macroscopically phase-separated solutions of PEG and sodium citrate (Figure 3). The citrate-rich phase contained the enzymes glucose oxidase (28:1 concentration excess compared to the PEG-rich phase) and horseradish peroxidase (1.6:1), as well as high concentrations of glucose (1.9:1) and peroxide (1.65:1). The hydrophobic substrate, amplex red and the
reaction product, resorufin strongly partitioned into the PEG-rich phase. First, resorufin formation (pink color) was observed in the citrate-rich phase, and after 10 min also at the interface. The reaction product continuously partitioned into the PEG-rich phase, so that after 3 h the PEG-rich phase was uniformly pink [23]. Fabrication of fibrillosomes through growing amyloid fibrils from hen egg lysozyme as monomer at all-aqueous interfaces seeded with pre-formed lysozyme fibrils also holds a merit [34].

**Microfluidics for all-aqueous emulsions**

Microfluidic emulsification enables fabrication of highly monodisperse all-aqueous emulsions. Due to the inherently laminar flow conditions in the small channels, the droplet formation is highly repeatable and can be precisely controlled at low energy input [35,36]. Application of microfluidics for generation of all-aqueous emulsions is rapidly developing. Because of the very low interfacial tension of all aqueous emulsions, the droplet formation mechanism is rather different from those presented for other two-phase systems. For example, for oil in water emulsions, inflow of the water phase at the point of droplet formation is essential for droplet formation to take place, and this occurs through the ‘gutters’ that form at the edges/corners of the rectangular channel [37–39]. For ATPSs, destabilization takes place through shear forces, which leads to destabilization of the neck which can perfectly take place in round channels.

Development of non-planar microfluidic channels is ongoing and should be taken in consideration for future all-aqueous emulsion production [40].

Recently, a microfluidics platform to make double and triple all-aqueous emulsions based on hybridization of a conventional microfluidic flow-focusing geometry with a coaxial microneedle and a glass capillary embedded in flow-focusing junctions was presented for making Dex-in-PEG-in-Dex emulsions [41]. Fabrication of such
microfluidic devices, however, is sometimes cumbersome and not easily available for scientists without a background in microfabrication. Recent developments in microfluidic fabrication have brought this out of the clean room, using 3D printers, laser cutters and sacrificial mold to create microchannels [42–45]. One example is the Embedded Scaffold RemovinG Open Technology (ESCARGOT), a simple and versatile 3D printing method which allows to make multilayered and intricate micrometric channels in a single block of polydimethylsiloxane (PDMS) [46]. This can be combined with various components such as heating units, color sensors and even a solenoidal micro-coil allowing NMR-spectroscopy in flow [46] for example, for the quantification of reactions in flow [47].

Due to the small amounts of reactants, analytical detection and reaction monitoring (on single droplet level) are usually challenging. Still, some options are available that use optical detection (mostly fluorescence), electrochemical signals, but also Mass Spectrometry and NMR [48]. As analytical devices are improving in sensitivity and specificity year after year, the detection of small-scale reactions will improve along with the analytical devices. In-line ultrasound-enhanced Raman spectroscopy for suspensions [49] and in-line nuclear magnetic resonance (NMR) embedded into flow reactors [50] are instances of the analytical techniques which can be used for monitoring reactions in microfluidic cells.

Concluding remarks

All-aqueous emulsions are a promising tool for running bioconversion reactions, synthesis of bio-microgels and bio-microcapsules. The main advantages of using all-aqueous emulsions as microreaction vessels include avoiding the utilization of synthetic
surfactants, and organic solvents. Moreover, the emulsion fabrication is readily achieved on large scale without need to high-pressure and high-speed homogenizers.

Although the application of all-aqueous emulsions for microgel synthesis is already advanced, performing reactions at the W-W interfaces is still in its infancy. The W-W interfacial reactions are promising for making food-grade core-liquid microcapsules, as well as to produce compounds that leave the interface and partition into either of the aqueous phases.

CRediT author statement

Ashkan Madadlou: Conceptualization, Writing- Original draft preparation
Vittorio Saggiomo: Writing- Original draft preparation
Karin Schroën: Writing- Reviewing and Editing
Vincenzo Fogliano: Writing- Reviewing and Editing

Conflict of interest. The authors declare no conflicts of interest.

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• of special interest

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• A strategic review on Particle-stabilized and segregatively phase separated biopolymer mixtures.


A pioneering and unique report on accomplishing enzymatic/chemical reactions at the water-water interface that stays liquid.


• The paper addresses microfluidic techniques for emulsion preparation. Both scale-up of standard microfluidic devices, and characterisation of emulsion formation and stability using purpose built microfluidics are discussed.


• A recent review on droplet microfluidics, from fabrication to analysis and future applications.


Figure 1. Schematic illustration of the setup used for formation of pollen-like alginate microparticles. The alginate-containing Dex droplets are generated inside the outlet tubing, and flow into a bath of PEG and CaCl₂ for 30 s. The polymerization and growth of spikes across the DEX–alginate droplets proceed over time. Scale bar indicates 50 μm [29].
Figure 2. **A)** Schematic illustration of the formation of bicomponent (dual component) protein particles in all-aqueous emulsion (a); Optical microscope images of hemoglobin–bovine serum albumin microparticles taken at the (b) beginning and (c) end of the fabrication process; Cryo-scanning electron microscopy image of the microparticles (d). Scale bar: 100 μm [30].

**B)** Scheme of the self-assembly of polyelectrolytes at the W-W interface and confocal microscopy images of the microcapsules, scale bar: 50 μm [31].

**C)** Optical images of the microfluidic device (a), the taper showing droplet movement when the pump was turn on (b), and off (c), and Dex droplets containing Na-alginate moving in the outer tube (d). The bottom scheme illustrates the fabrication mechanism of Ca-alginate microgels through urease-mediated biomineralization (CaCO₃ generation) at the Dex-alginate phase interface [33].
**Figure 3.** Progress of the reaction between amplex red and hydrogen peroxide in the presence of horseradish peroxidase at the W-W interface between a citrate-rich aqueous phase and a PEG-rich aqueous phase [23].
Table 1. Examples of the bio-compounds segregated using ATPSs.

<table>
<thead>
<tr>
<th>ATPS pair</th>
<th>Segregated compound</th>
<th>Host phase</th>
<th>$K_p$; RE</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCON–KH$_2$PO$_4$</td>
<td>Laccase</td>
<td>KH$_2$PO$_4$</td>
<td>$&gt;3$</td>
<td>[17]</td>
</tr>
<tr>
<td>PEG–Na$_2$SO$_4$</td>
<td>Laccase</td>
<td>Na$_2$SO$_4$</td>
<td>$\sim 100%$</td>
<td>[51]</td>
</tr>
<tr>
<td>PEG–Na$_3$PO$_4$</td>
<td>Recombinant chymosin</td>
<td>PEG</td>
<td>4-6</td>
<td>[21]</td>
</tr>
<tr>
<td>PEG–K$_3$PO$_4$</td>
<td>Gallic acid, Epicatechin, Chlorogenic acid</td>
<td>PEG</td>
<td>1.01-4.6, $&gt;75%$</td>
<td>[52]</td>
</tr>
<tr>
<td>PEG$<em>{113}$-b-PNIPAM$</em>{149}$–Salt</td>
<td>Bromelain</td>
<td>PEG$<em>{113}$-b-PNIPAM$</em>{149}$</td>
<td>$\sim 95%$</td>
<td>[53]</td>
</tr>
<tr>
<td>PEG–Dex</td>
<td>Immunoglobulin monomers</td>
<td>Either PEG or Dex</td>
<td>–</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>Immunoglobulin aggregates</td>
<td>Interface</td>
<td>$\sim 100%$</td>
<td></td>
</tr>
<tr>
<td>Ethanol–K$_2$HPO$_4$</td>
<td>Vanillin</td>
<td>Ethanol</td>
<td>$\sim 96%$</td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>K$_2$HPO$_4$</td>
<td>92–2%</td>
<td></td>
</tr>
</tbody>
</table>

$K_p$: Partition coefficient  
RE: Recovery extent (%)  
UCON 50-HB-5100: A random copolymer of ethylene oxide and propylene oxide.  
PEG$_{113}$-b-PNIPAM$_{149}$: Block copolymer of PEG (mean degree of polymerization: 113, $M_n = 5.0$ KDa) and poly (N-isopropylacrylamide) (PNIPAM); LCST 28.6°C.