

# Cell-Free Protein-Synthesis Systems in Printed Matrices: Reagent State, Activation, Transport and Functional Lifetime

*Engineering cell-free expression as a material function in structured hydrogel and photopolymer matrices*

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## Abstract

Cell-free protein synthesis can be embedded in hydrated matrices so that transcription and translation occur without living cells. For printed or photocurable structures, the critical engineering variables are reagent preservation, activation, water and nutrient transport, product diffusion, matrix compatibility and functional lifetime. This review distinguishes cell-free activity from living-cell viability, defines the material state before and after activation, and proposes a qualification framework for output, spatial distribution, reuse and storage. It does not treat whole-cell biosensing, which is addressed separately in ER-436.

Keywords: cell-free protein synthesis; hydrogel; printed matrix; transcription; translation; activation; diffusion; functional lifetime

## 1. Significance and scope

Cell-free protein synthesis (CFPS) decouples gene expression from living-cell maintenance, allowing the reaction machinery to be embedded directly in a material. Whitfield and co-workers demonstrated macro-scale hydrogel materials in which CFPS occurred without an external liquid phase, establishing that a hydrated matrix can act as a physical chassis for expression [1]. The qualification problem is therefore one of reagent state, transport and lifetime rather than viability.

A CFPS material should be described by the expression system as well as by the polymer matrix: extract source, DNA or RNA template form, energy system and feeding regime can all change output. Because no living cell repairs or replenishes damaged machinery, processing damage to ribosomes, enzymes or template is carried directly into the functional lifetime of the material. The matrix can protect components, but it can also immobilise them in an inaccessible state.

## 2. Reagent state before activation is part of material identity

Extracts, DNA templates, ribosomes, cofactors and energy substrates can lose activity during formulation, drying or storage. The pre-activation state should therefore be defined: wet, freeze-dried, separately reconstituted or continuously fed. Sánchez-Costa and co-workers showed that freeze-dried agarose systems could retain the ability to synthesize and release protein after reconstitution, demonstrating that storage state and activation protocol can be engineered explicitly [2].

Storage qualification should separate preservation of the genetic template from preservation of the translation machinery. The PEGDA/DNA system of Cui and co-workers demonstrates repeated use of an immobilised DNA template [3], whereas freeze-dried extract-containing hydrogels address preservation of a broader biochemical system [2]. Those are different claims and require different failure metrics.

Template accessibility should also be separated from template integrity. DNA can remain chemically intact while becoming sterically inaccessible, strongly adsorbed or locally concentrated in a way that changes transcription kinetics. Repeated-use claims therefore need both expression output and evidence that the immobilised template remains available over cycles. Cui and co-workers provide a useful example of DNA retained within a PEGDA hybrid matrix, but transfer to a different network requires rechecking mesh size, charge interactions and rehydration history [3].

## 3. Polymerisation and matrix chemistry can suppress or enhance expression

A matrix can concentrate DNA or proteins, restrict diffusion, alter ionic strength or adsorb essential components. PEGDA/DNA hybrid hydrogels have been used to retain DNA templates and support repeated expression, showing that the polymer network can directly alter CFPS efficiency [3]. For photocurable structures, cure conditions should be assessed for their effect on both the template and the translational machinery.

Activation kinetics should include hydration time, reagent penetration and the delay to detectable product. A construct that eventually produces a large amount of protein can still be unsuitable for rapid on-demand use if reconstitution is diffusion-limited. Geometry should therefore be varied or modeled when comparing activation times between formulations.

A useful activation protocol has to define the moment at which functional lifetime begins. If dried matrices are rehydrated gradually, different regions may start expression at different times, producing an apparent long-lived output that is actually asynchronous activation.

Spatially resolved reporter measurements, coupled where possible to local hydration or tracer penetration, help distinguish prolonged biochemical activity from delayed reagent access.

#### 4. Transport controls both feed and product recovery

CFPS consumes small molecules and produces proteins whose diffusion depends on size and matrix mesh. A strong fluorescent signal inside the gel is not proof that product can be delivered outside it, and rapid release is not proof that synthesis occurred uniformly throughout the matrix. Spatial expression imaging, product recovery and transport characterization should be treated as separate measurements.

Product transport should be quantified independently from synthesis. Fluorescent reporter accumulation inside a gel can overestimate delivered output if the protein is trapped, while rapid release can reduce the local signal without reducing synthesis. Paired measurements of internal and released product, or a mass balance where feasible, distinguish expression capacity from delivery performance.

Product quality matters as much as quantity. Fluorescent reporter intensity can show that translation occurred but does not establish correct folding or biological activity for a therapeutic, enzyme or regulatory protein. Where the claimed function depends on protein activity, at least one activity assay should be performed on material-generated product. This is especially important after photocure, drying or prolonged storage because these steps can preserve expression while altering the chemical environment experienced by the product.

#### 5. Functional lifetime differs from storage lifetime

A material can remain activatable after storage yet have a short active expression period after hydration. Conversely, a continuously fed system can sustain expression long after a closed batch exhausts energy substrates. Ouyang and co-workers showed prolonged protein synthesis in hydrogel-based artificial cells when energy and nutrients were continuously supplied, illustrating the importance of specifying the feeding regime [4].

Functional lifetime is also sensitive to by-product accumulation and energy depletion in closed systems. Continuous or intermittent feeding changes the thermodynamic and transport boundary conditions, so batch and flow-fed expression should not be compared as if they were the same material. The Ouyang study illustrates why the feeding architecture belongs in the specification of sustained CFPS [4].

| Axis          | Measurement                                 | Failure mode                                   |
|---------------|---------------------------------------------|------------------------------------------------|
| Reagent state | wet/dry state, storage time and temperature | activation loss before use                     |
| Activation    | medium, hydration and feeding protocol      | undefined start of functional life             |
| Expression    | yield and product identity/activity         | fluorescence without functional protein        |
| Transport     | feed and product diffusion/recovery         | internal synthesis with inaccessible output    |
| Lifetime      | active duration and reuse/feeding mode      | storage stability confused with operating life |

**Table 1.** Domain-specific qualification matrix for cell-free protein-synthesis systems in printed matrices.

#### 6. Device-level verification

The final printed geometry should be activated using the intended protocol and tested for expression yield, spatial uniformity, product activity, release or retention and duration. If the structure is intended as a sensor, response time and background expression are required; if intended as a producer, volumetric productivity and recovery are more relevant. Repeated activation should be distinguished from continuous operation.

ER-436 covers living-cell biosensing and is not interchangeable with CFPS sensing or production. A cell-free matrix can implement a genetic circuit without any viability requirement, but its calibration must still state reagent state, activation history and depletion because the signal-generating machinery itself ages. Stability testing should report both time-to-activation and post-activation output.

Dry storage at ambient or refrigerated conditions, humidity excursions and repeated reconstitution may affect different components. A reference aliquot of the same CFPS mixture in solution can help distinguish matrix-protective effects from ordinary lot-to-lot changes in extract activity.

#### 7. Minimum qualification framework

Report CFPS system origin, template identity, matrix chemistry, cure or gelation process, pre-activation storage state, activation medium, geometry, expression output, product activity, spatial distribution, diffusion/release and functional lifetime. Controls should establish that the measured product is synthesized after activation rather than produced before gelation and merely trapped. Qualification should identify the output basis: total protein, active protein, reporter fluorescence or released product.

Fluorescence alone can be distorted by maturation kinetics or matrix optics, and total protein does not establish activity for enzymes or therapeutics. Where function matters, an activity assay should accompany expression yield. The central engineering claim is not merely that translation occurs, but that the stored and activated matrix delivers a reproducible functional output over a declared time window.

## 8. Engineering conclusions

Cell-free expression turns a printed matrix into a biochemical reactor whose function begins at activation. Qualification should therefore track the full state transition from stored reagent-containing material to hydrated active reactor and finally to depleted or deactivated state. The useful design window is defined by preserving the molecular machinery during fabrication while still allowing enough transport to support expression and deliver the intended output.

## References

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## Competing interest

Juan Segurola is founder, CEO and CTO of 3Dresyns by Resyner Technologies S.L., which develops and commercialises photopolymer materials and related additive-manufacturing technologies. This review is an engineering analysis and does not constitute a product-performance claim or a device qualification.