Synthetic transient crosslinks program the mechanics of soft, biopolymer based materials

Jessica S. Lorenz\textsuperscript{1,3}, Jörg Schnauß\textsuperscript{1,2,3,*}, Martin Glaser\textsuperscript{1,2}, Martin Sajfutdinow\textsuperscript{1}, Carsten Schuldt\textsuperscript{1,2}, Josef A. Käs\textsuperscript{2}, David M. Smith\textsuperscript{1,*}

\textsuperscript{1}Fraunhofer Institute for Cell Therapy and Immunology (IZI), 04103 Leipzig, Germany
\textsuperscript{2}Leipzig University, Peter Debye Institute for Soft Matter Physics, 04103 Leipzig, Germany
\textsuperscript{3}These authors contributed equally to this work.

((Optional Dedication))

Dr. J. S. Lorenz\textsuperscript{1,3}
\textsuperscript{1}Fraunhofer Institute for Cell Therapy and Immunology (IZI), 04103 Leipzig, Germany
\textsuperscript{3}These authors contributed equally to this work.

Dr. J. Schnauß\textsuperscript{1,2,3,*}
\textsuperscript{1}Fraunhofer Institute for Cell Therapy and Immunology (IZI), 04103 Leipzig, Germany
\textsuperscript{2}Leipzig University, Peter Debye Institute for Soft Matter Physics, 04103 Leipzig, Germany
\textsuperscript{3}These authors contributed equally to this work.
\textsuperscript{*}E-mail: joerg.schnauss@uni-leipzig.de

M. Glaser\textsuperscript{1,2}
\textsuperscript{1}Fraunhofer Institute for Cell Therapy and Immunology (IZI), 04103 Leipzig, Germany
\textsuperscript{2}Leipzig University, Peter Debye Institute for Soft Matter Physics, 04103 Leipzig, Germany

M. Sajfutdinow\textsuperscript{1}
\textsuperscript{1}Fraunhofer Institute for Cell Therapy and Immunology (IZI), 04103 Leipzig, Germany

C. Schuldt\textsuperscript{1,2}
\textsuperscript{1}Fraunhofer Institute for Cell Therapy and Immunology (IZI), 04103 Leipzig, Germany
\textsuperscript{2}Leipzig University, Peter Debye Institute for Soft Matter Physics, 04103 Leipzig, Germany

Prof. Dr. J. A. Käs\textsuperscript{2}
\textsuperscript{2}Leipzig University, Peter Debye Institute for Soft Matter Physics, 04103 Leipzig, Germany

Dr. D. M. Smith\textsuperscript{1,*}
\textsuperscript{1}Fraunhofer Institute for Cell Therapy and Immunology (IZI), 04103 Leipzig, Germany
\textsuperscript{*}E-mail: david.smith@izi.fraunhofer.de

Keywords: biomimetic materials, biopolymers, DNA nanotechnology, molecular designs, crosslinkers
Abstract

Actin networks are adaptive materials enabling dynamic and static functions of living cells. A central element for tuning their underlying structural and mechanical properties is the ability to reversibly connect, i.e. transiently crosslink, filaments within the networks. Natural crosslinkers, however, vary in every parameter and systematically studying the impact of their fundamental properties like size and binding strength is limited by the inability to independently tune these structural parameters. Here, this problem is circumvented by employing a modular strategy to construct purely synthetic actin crosslinkers from DNA and peptides, which mimic both intuitive and non-canonical mechanical properties of their natural counterparts. By isolating binding affinity as the primary control parameter, effects on structural and dynamic behaviors of actin networks are characterized. A concentration dependent triphasic behavior arises from both strong and weak crosslinkers due to emergent structural polymorphism. Beyond a threshold, strong binding leads to a non-monotonic elastic pulse marked by an initial burst and exponential decay, which self-destructs the mechanical structure of the underlying network. The modular design also facilitated an orthogonal regulatory mechanism based on enzymatic cleaving. This approach can guide the rational design of further biomimetic components for programmable modulating the properties of biomaterials and even cells.
Living cells exhibit an elaborate commixture of both dynamic and stabile material behaviors, which enable them to rapidly adapt to complex environments\cite{1}. The filamentous, cytoskeletal biopolymer actin is at the heart of this ability, simultaneously forming load-bearing networks, mechanosensing protrusions, and motility-inducing ratchets\cite{1-3}. Actin-associated proteins that form transient, physical crosslinks between filaments have been implicated in nearly every actin-dependent process and their role in directing structural morphology, mechanical stasis, and active behaviors has been well documented\cite{1}. In contrast to permanent, chemical crosslinks in classical polymer systems, these biological crosslinks have a far broader parameter space defined by the molecular details of their binding and connective regions. Factors such as on-/off-rates, size, flexibility, orientation, and biochemical switches allow these components to exert control over the material properties of both networks and cells beyond simply imposing local affine responses against external deformations, which renders actin based structure highly adaptive\cite{2,4-7}.

The resulting richness of structural phases in soft, polymeric materials has been captured in a theoretical framework built upon the classical Onsager approach describing isotropic-nematic phase transitions in rigid rods, where transient crosslinks are modelled as distance-dependent interrod attractive potentials\cite{8}. In contrast to the established triphasic (isotropic, nematic, coexistence) character for noninteracting rods, the addition of nonpermanent crosslinks revealed a highly complex, polyphasic behavior dependent upon the energetic preference for parallel or perpendicular orientation between linked filaments\cite{8}. A complex mixture of states more closely resembling both experimental observations and biological structures is predicted in analytical and simulation based studies, with stress fiber-like bundles and regular, cubatic networks commixing with the previously known isotropic and nematic phases\cite{8-10}.

Still, a critical discrepancy remains; the coexistence of phases favored by parallel and perpendicular binding preference should only occur in an unphysiologically narrow range\cite{8}.

However, the coexistence of bundles and regular networks are ubiquitous in mixtures of
biopolymers such as actin and naturally occurring transient crosslinker α-actinin, possibly due to the low binding affinity of the binding interaction \cite{8,11–13}. Likely, molecular and physical details of the biological constructs enable the structural polymorphism of actin that is important to the mechanical behaviors of cells. This, however, imposes a central limitation to systematic investigations and development of biomimetic or bio-inspired materials: using different native biological crosslinkers to explore the phase space of one parameter (e.g. using α-actinin and fascin to vary binding strength) is unavoidably coupled with unwanted variations of several other parameters \cite{14,15}. Genetic mutation can be used to perturb crosslinker properties such as binding kinetics or size \cite{16–18}, however, its empirical nature and complexity inhibits systematic studies where different key parameters can be modified in a completely decoupled way.

Here, we resolve this natural limitation by constructing synthetic actin crosslinkers based on a well-defined DNA template. The core building block is a segment of double-stranded DNA (60 bases \( \equiv \) length of 20 nm), which can be covalently attached to actin binding peptides via copper-free click chemistry (Figure 1a, see Experimental section) \cite{19}. This allows us to conserve the basic geometry of the crosslinkers while independently altering the affinity of the binding domains to actin to examine the impact on structural and bulk mechanical properties. We used this plug&play platform to conjugate either the linear, 17-residue actin binding peptide commercially known as LifeAct® (\( K_D(F-{\text{Actin}}) \approx 2.3 \, \mu M \pm 0.9 \, \mu M \)) \cite{20} or the bicycllic heptapeptide phalloidin (\( K_D(F-{\text{Actin}}) \approx 9 \, nM \pm 2 \, nM \)) \cite{21} to both ends of the DNA template to mimic weak or strong binding, respectively (see also Figure S1) \cite{20,21}. For simplicity, we henceforth denote the weak LifeAct®-based and strong phalloidin-based crosslinking constructs as wLX and sPX, respectively. To quantitatively evaluate emergent effects of these two crosslinkers, we employed dynamic shear rheology to test a broad range of crosslinker concentrations (ratio \( R = c_{\text{crosslinker}}/c_{\text{actin}} \)) in viscoelastic actin filament networks (see Experimental section). These biomimetic crosslinkers reconstruct many of the trivial
morphological and rheological behaviors consistent with actin networks crosslinked by native proteins. Moreover, we find that imposing a strong binding energy leads to the time-dependent build-up and subsequent decay of pre-stress within the network, a striking signature of soft glassy materials previously seen in actin networks tightly bundled by fascin\cite{14,22}. Finally, a regulatory mechanism based on the enzymatic severing of the core template was demonstrated as an orthogonal "off-switch" of crosslinking activity.

Figure 1. Constructing synthetic actin crosslinkers. a) A copper-free click chemistry approach\cite{19} is used to covalently attach two actin-binding peptides to double-stranded DNA. After hybridization of the complementary strands, Dibenzocyclooctyne-NHS ester covalently reacts with primary amine groups on each end of the double-stranded DNA-spacer. Subsequently, an azide-containing actin binding peptide forms a covalent bond with the previous attached DBCO group. b) Since the two binding domains are connected by double-stranded DNA, the construct can physically crosslink actin filaments within the system.
The elasticity (G') of polymerizing actin solutions increased monotonically over time, which is the expected signature of the elongating filaments arranging into an isotropic, entangled network. Once the steady state of actin polymerization is reached the elasticity remains stable over time (Figure 2a). These qualitative characteristics were conserved when including the weakly binding wLX in the polymerizing actin networks, although accompanied by the additional emergence of crosslink-dependent effects. The overall elasticity displayed a notable 6-fold increase compared to pure actin for higher wLX concentrations of 2.4 µM (R = 0.1) illustrating the effect of physically, albeit transiently connecting individual filaments, which has been reported previously for the weakly binding natural crosslinker α-actinin\[14,23\]. For the high-affinity sPX, a similar behavior is observed for comparatively lower concentrations, with a six-fold increase of G' seen at a molar ratio of R = 0.01, or approximately 10 % of the concentration of wLX required to reach a similar magnitude of elasticity (Figure 2b – blue curves). In comparison to pure, viscoelastic actin networks, which display a phase angle δ around 40 ° (with δ defined as the arctangent of the ratio G’’/G’, where G’’ and G’ are the frequency dependent viscous and elastic shear moduli, respectively, with 0° being a purely elastic (solid-like) response and 90 ° being a purely viscous (i.e., Newtonian fluid) response), the phase angle for both types of crosslinked networks is shifted to lower values, indicating their increasing elastic response (Figure 2c – blue curves). Once again, equivalent behavior can be found in the natural analogue, where these signatures of gelation have been reported for similarly low ratios of the high-affinity natural crosslinker human fascin\[14\].

However, this mechanical fingerprint drastically changes at higher sPX concentrations: when reaching a threshold of R values between 0.01 and 0.02, the time evolution of G’ becomes non-monotonic, marked by an initial "burst" that relaxes to a viscous dominated state (Figure 2b – green curves). The nature of this elastic pulse is further revealed through the concurrent evolution of the phase angle δ over time (Figure 2c – green curves). Before the peak of G’ is reached, δ remains stably low and the system exhibits a predominantly elastic response. In the
subsequent regime of decaying $G'$, $\delta$ monotonically increases before reaching a stable plateau, corresponding to the emergence of an increasingly viscoelastic response. Interestingly, for the highest sPX concentration tested (Figure 2c – dark green curve), $\delta$ reaches significantly higher values than for solutions of purely entangled actin filaments (Figure 2c - black curve), and the system predominantly behaves as a viscous fluid.

This dynamic pulse behavior closely resembles previously reported findings as well as our own measurements for the natural strongly-binding actin crosslinker fascin (supplemental Figure S7)\textsuperscript{[14,22]}. Simultaneous polymerization and strong crosslinking has been shown to generate pre-stressed structures that are bent beyond their equilibrium configuration\textsuperscript{[22]}, and accordingly the forming actin networks build up internal stress\textsuperscript{[22,24]}. This occurs because the typical filament elongation rates ($\sim 10 \, \text{µM}^{-1} \, \text{s}^{-1}$)\textsuperscript{[25]} far exceed the off rates of the actin-binding domains (0.12 s$^{-1}$ for fascin\textsuperscript{[26]}, 0.00026 s$^{-1}$ for phalloidin\textsuperscript{[21]}), which is the kinetic mechanism primarily responsible for relaxing sterically trapped non-equilibrium configurations. The subsequent relaxation is likely induced by glassy dynamics aiming to release the stress towards equilibrium\textsuperscript{[22]} and breaking of filaments subjected to significant bending forces\textsuperscript{[27]}.

The stochastic unbinding of a crosslink from a filament is followed by its subsequent rebinding in a more relaxed configuration, when the network contains less internal stress driving the decay\textsuperscript{[11]}. This in turn gradually decreases the unbinding probability with increasing network age\textsuperscript{[11,22]}. This suggested deceleration in the change of the network’s elasticity is supported by the exponentially decaying relaxation behavior in Figure 2b & d.

However, the increasingly dominant fluidization observed from the phase angle at the high-sPX limit cannot be solely explained by relaxation of prestress, but likely also reflects the mesoscale structural evolution within the network. We hypothesize that short, oligomeric actin filaments formed at the onset of the polymerization are almost instantaneously crosslinked to other such protofilaments in their entropically favored, parallel bundled configuration. These bundles grow due to actin polymerization while simultaneously
depleting the surrounding monomer pool. These simultaneous processes can even be enhanced by the concentration-dependent tendency of crosslinkers to suppress actin depolymerization kinetics\textsuperscript{[28]}. Initially, this leads to the observed burst of $G'$ as the rapidly elongating bundles merge due to high crosslink concentrations, and become percolated throughout the network, sterically trapped in pre-stressed configurations\textsuperscript{[22,24]}. The pool of actin monomers is eventually depleted to its steady-state concentration, halting further elongation of the constituent filaments. Beyond a critical turning point, bundles become increasingly disconnected from the rest of the network due to the energetic preference of strong crosslinkers for binding of parallel pairs of filaments\textsuperscript{[9,10]}. This is possibly enhanced the breakage of filaments under bending and torsional stress\textsuperscript{[27]}. This depercolation leads to the observed drop in $G'$ and the simultaneous increase in the loss modulus $G''$ (and accordingly $\delta$) shown in Figure 2c. This transition process is supported by prior Monte Carlo simulations, which suggested that bundle-dominated networks contain a larger number of crosslinkers forming bridges between parallel filaments within a single bundle, and fewer contributing to overall network percolation as the degree of bundling becomes more pronounced\textsuperscript{[10]}. \hspace{1cm}
Figure 2. Time evolution of the elasticity during actin polymerization in presence of synthetic actin crosslinkers.

a) Mechanical properties of actin networks (24 µM) enriched with different concentrations (R = 0, 0.001, 0.01, 0.1) of the wLX were determined via dynamic shear rheology. Measurements always started with monomeric actin and the monotonically increasing G’ of pure actin (black) illustrates the formation of filaments and their arrangements into entangled networks. Upon addition of the crosslinker, the elasticity increased in a concentration dependent manner similar to the natural crosslinker α-actinin (accordung crosslinker concentrations are given in different shades of red). 

b) Strong phalloidin crosslinkers induced entirely different mechanical fingerprints comparable to findings reported for the strong natural crosslinker fascin[14,22]. Networks are initially monotonically stiffened similar to wLX, but at much lower concentrations (R = 0, 0.0025, 0.01, 0.02, 0.1). When reaching a concentration threshold, the time evolution of G’ becomes non-monotonic. First, the elasticity drastically increases until reaching a peak, which is subsequently followed by an exponential decay - a time evolution which is referred to as the “elasticity burst”[14].

c) The corresponding phase angles illustrate that low crosslinker concentrations (blue curves) induce a predominantly elastic response. High crosslinkers densities initially induce a predominant elastic response (green curves), but in the time evolution the systems become increasingly viscous.

d) The higher sPX concentration (dark green) induces the elasticity burst earlier with a higher magnitude of G’ with a fast subsequent relaxation indicated by the shorter decay time. Inset: Similar characteristic fingerprints have been observed in actin networks with a high concentration of the natural crosslinker fascin[14].

We hypothesize that the peak of the elasticity burst is reached faster for the highest sPX concentration (R = 0.1) due to enhanced depletion of the monomer pool[28] and trapping of prestressed structures during polymerization. The characteristic decay time τ significantly increases for lower sPX concentrations, and this non-monotonic effect disappears altogether for sPX concentrations below R = 0.01 (Figure 2d). The according decay times are comparable to a previous study on fascin employing the same crosslinker/actin concentrations.
as in our study (Figure 2d inset)\(^{[14]}\). In contrast, the lower actin concentrations utilized by Lieleg et al. resulted in a much longer decay time \(\tau\) for fascin induced elasticity bursts (supplemental Table S3)\(^{[22]}\), likely due to combined effects of (a) smaller bundles inducing a lower collective restoring force\(^{[29,30]}\) and (b) fewer bundles driving depercolation during the relaxation process. Similar to actin/fascin networks\(^{[14,22]}\), the final steady-state \(G'\) is lower in cases where an elasticity burst occurs than the steady-state \(G'\) where no burst is observed as well as for uncrosslinked networks. Since we are using the same basic geometries for our different synthetic crosslinkers, the induced elasticity burst appears to be a general feature of crosslinkers with low off-rates and is seemingly independent of other parameters such as their length or structure.

This pulse-like nature of the initial elasticity burst effectively acts as a reporter signal of the material's molecular constituents. Rather than imposing inherent, static material properties like strain hardening or fixed shear moduli, this effectively acts as a rudimentary bio-hybrid signal processor, where both the amplitude and width/decay time of the pulse are determined by the biopolymer and crosslinker densities (Table S3). While the relaxation reported here is effectively an irreversible transition due to the loss of mechanical structure, the molecular constituents in the system - namely actin proteins and DNA-peptide crosslinkers - are themselves likely unharmed by the non-chemical nature of the decay. Therefore, this points towards a possible strategy for engineering soft, mechanical transducers, based on the recyclable, self-destruction of network's underlying mesostructure.

These findings already indicate that the formation of different morphologies such as networks and/or bundles leads to non-trivial mechanical characteristics, which highly depend on the crosslinker type and concentration. For both sPX and wLX, we found a qualitatively similar, non-monotonic, concentration dependent behavior of the network elasticity (Figure 3a). For the lowest respective concentrations, \(G'\) was slightly lower compared to purely entangled actin networks. In this regime, the network is not percolated, but crosslinkers already induce
local heterogeneities triggering a global softening\textsuperscript{[5,31].} With increasing concentrations the network is progressively more percolated, leading to a rising elasticity as illustrated by the positive slopes in Figure 3a. However, comparing these slopes for the different crosslinker binding affinities reveals that sPX stiffen the network much more efficiently than wLX, displaying a power law exponent of 0.62 compared to 0.21 for the weaker case.

Interestingly, both cases exhibit a local maximum of $G'$, which subsequently decreases to a minimum and rises again with a different exponential behavior for the highest concentrations. Although the onset of the different regimes varies, the qualitative curve progression for both binding strengths resemble each other in appearance. Generally, this non-monotonic fingerprint can be attributed to different structural morphologies induced at the according crosslinker concentrations\textsuperscript{[5].} In the following explanation, the observed tri-phasic behavior is described for networks containing wLX, however we expect, and prior findings indicate\textsuperscript{[5,15]}, that the characteristics will be generally valid for other synthetic and natural actin crosslinkers.

In the isotropic regime (I) occurring at low wLX concentrations ($R \leq 0.01$), actin filaments are arranged in a weakly crosslinked network. With increasing wLX concentrations, networks remain mostly isotropic, as illustrated by the constant light scattering intensity (Figure 3b – blue curve) and visually shown in Figure 3c. Above a threshold concentration, the network enters the coexistence regime (II), where higher wLX densities increasingly favor alignment of filaments, yielding a coexistence of bundles within an isotropic background network and a concentration-dependent decrease of $G'$. This new structural arrangement is captured by the sudden change of the light scattering profile at the onset of the elasticity decrease (Figure 3b) and is directly observable with fluorescence microscopy (Figure 3c). In this regime ($0.01 < R \leq 0.08$), bundles can be considered as local anisotropies, thus the effective actin concentration in the percolated background network is reduced weakening the overall structure and resulting in the observed decrease of $G'$. A study based on Monte Carlo simulations emphasized that bundled configurations occupy more crosslinkers than their
isotropic counterparts suggesting that this increasing anisotropy comes at the cost of overall network percolation\textsuperscript{[10]}. This combination of both reducing the effective actin concentration and concurrently reducing percolation in the background network accounts for the drastic decrease of $G'$ to values even below those found for much smaller crosslinker concentrations\textsuperscript{[10]}. Finally, at high wLX ratios ($R > 0.8$) the elastic response is dominated by a network of percolated bundles (III). Here, higher crosslink densities continue to favor an increasing prevalence of bundled structures (Figure 3c), which is also illustrated by the rising light scattering intensity. Accordingly, the elasticity increases monotonically. This qualitative structural trend can also be seen in networks containing sPX although light scattering analysis is limited due to the time dependent evolution of structural anisotropies, but clear differences become apparent when inspecting the sample with fluorescence microscopy (see Figure S8).

Figure 3. Synthetic crosslinkers non-monotonically influence the elasticity of actin networks. a) Elastic moduli of actin networks were investigated for different concentrations of wLX (red) and sPX (green) crosslinkers. Both initially stiffen the network with increasing concentration before reaching a peak, which is followed by a decrease of $G'$ to a minimum with a subsequent a monotonic increase (power law fits (black line) and exponents are shown next to the according $G'$ increase). This non-monotonic behavior can be attributed to different structural morphologies, which are captured by b) light scattering (blue; error bars indicate standard deviations of the mean values) and c) fluorescence microscopy.
This structural polymorphism has been previously observed for natural crosslinkers\textsuperscript{[5,32]} and is supported by coarse-grained molecular dynamics\textsuperscript{[8,33]} and Monte Carlo\textsuperscript{[9,10]} simulations. In study by Borukhov et al., modelling crosslinkers as an effective rod-rod attraction within a variant of the classical Onsager theory reveals a rich phase space including networks, bundles, and their coexistence\textsuperscript{[8]}. Interestingly, the measured slopes for increasing G’ vary differently between regimes I & III for the two crosslinkers. The stronger actin-binding affinity of sPX induces a roughly three-fold steeper increase in regime I than the comparatively weak interaction of wLX, likely due to the longer binding times and thus more efficient percolation of the network. However, the trend is inverted in regime III: wLX induces a roughly 2.6-fold higher concentration-dependent increase in G’ than sPX. Since unbinding kinetics of phalloidin are much slower compared to LifeAct\textsuperscript{®}, we expect that sPX have an energetic preference to be stored in multi-filament bundles, and thus fewer crosslinkers contribute to the percolation of the system consistent with previous simulations\textsuperscript{[10]}. Effectively, this would mean that strong-binding crosslinkers generally have a lower "percolation efficiency" in the presence of bundles than their weakly binding counterparts, accounting for the steeper concentration dependent increase of G’ for wLX in regime III.

In addition to mimicking natural mechanical signatures, our modular, DNA-based design also enables the integration of rudimentary regulatory mechanisms in the form of orthogonal state-switches. Here, the sequence for the DNA connector included a recognition site for the EcoRV restriction enzyme so that the crosslinker could be cleaved into two segments, thereby effectively "breaking" the link between filaments (see Methods section and Figure S2). Incubating crosslinkers with EcoRV prior to measurement resulted in a drastic decrease of the elastic modulus to values close to the actin-only control (Figure 4). Marginal differences are well within the sample-to-sample variation, likely resulting from small numbers of undigested crosslinkers (supplemental Figure 2B).
Figure 4. Reversibility of synthetic actin crosslinkers. a) The time-dependent elasticity of actin polymerization was investigated in presence of intact sPX (dark green, c = 0.24µM (R = 0.01)), i.e. below the threshold of the elasticity burst) and compared to EcoRV-HF®-cleaved sPX (light green, c = 0.24 µM (R = 0.01)). Cleaving the crosslinkers reversed the stiffening effect illustrating the possibility to switch between different mechanical states. The cleavage was additionally verified via native polyacrylamide gel electrophoresis (Figure S2) and has also b) a drastic effect on the macroscopic behavior of actin as shown in an inclined cuvette monitoring the bulk properties over time. Left row: Pure actin was polymerized at 24 µM. Middle row: Actin was polymerized at 24 µM with 9.6 µM sPX. Right row: Actin was polymerized at 24 µM, including 9.6 µM sPX and 300 units EcoRV-HF®.

The mechanical impact of crosslink-induced stiffening and enzyme-based regulation can also be seen in macroscopic gels. A purely entangled, viscoelastic solution of actin filaments flows down a gradient within seconds (Figure 4b, left column) while the addition of crosslinks amplifies the elastic behavior, slowing the flow by more than five-fold (Figure 4b, center). Enzymatically severing the crosslinks restores the dominance of the fluid-like contribution,
once again inducing flow effectively indistinguishable from the actin-only solution (Figure 4b, right).

The effects of the synthetic crosslinkers described here are striking examples of how soft, biopolymer based materials can be fundamentally altered by rationally designed molecular components. To our knowledge, these constructs are the first example of purely synthetic actin crosslinkers and facilitated a systematic study into the global impact of their binding affinity when it is decoupled from other structural parameters. Varying the interaction strength and concentration of the crosslinkers enabled us to attain a control over the actin network elasticity spanning more than two orders of magnitude. Remarkably, the two different synthetic crosslinkers closely reconstruct the mechanical fingerprints of analogous natural crosslinkers such as α-actinin and fascin. Similar to fascin, high concentrations of the strongly-binding, phalloidin-based crosslinker sPX induced a non-monotonic time evolution and out-of-equilibrium dynamics (elastic pulse) during the formation of actin networks\textsuperscript{[22,24]}. Since the natural strong crosslinker fascin and its synthetic analogue here are only similar in their high binding affinity to actin, this special time evolution seems to predominantly depend on binding strength and concentration. Additionally, different concentrations of both crosslinkers induced three distinct regimes for structural morphology and mechanical behavior, which have been also reported for natural crosslinkers\textsuperscript{[5]} and hypothesized in analytical and simulation approaches\textsuperscript{[8–10,33]}. Ultimately, the crosslinker/filament ratio and strength of the actin-binding domains impact the degree of bundling and percolation within the system, resulting in the observed non-monotonic concentration dependency of G’.

Beyond simply reconstructing behaviors arising from natural actin crosslinking proteins, this programmable toolbox gives an entry point for fabricating non-canonical modulators of bio-hybrid materials that are not constrained by typical biological function. This is not only limited to alterations of structural or physical parameters; additional components for stimulus-response mechanisms can be integrated. While we showed this in a very simple form through
the sequence-specific enzymatic severing of the crosslinker, more complex components such as aptamer-based logic gating, light- or pH-triggered conformation switches, complex transcription circuits or more can be implemented to convert a specific stimulus into a distinct structural or mechanical signature\textsuperscript{[34-37]}. This potentially serves as a foundation for strategies to design signal-responsive, programmable, smart biomaterials for cell-based or dynamic sensing applications, or as intracellular tools to synthetically model the impact of crosslink mutation in disease pathology\textsuperscript{[17]}. 
Experimental Section

**Actin preparation:** Globular actin (G-actin) was prepared from rabbit muscle as described previously\[38\]. Actin was polymerized by the addition of 8.75 µl 20x F-Buffer at a final concentration of 24 µM and a volume of 175 µl.

**Synthetic actin crosslinkers:** Lyophilized oligonucleotides (Table S1; biomers.net GmbH, Germany) were resuspended in Millipore water and concentrations were spectrophotometrically determined by a NanoDrop 1000 (Thermo Fisher Scientific Inc., USA). Complementary oligonucleotides were hybridized in 100 mM KH_2PO_4 pH 7.2 at a final concentration of 25 mM in a thermocycler (denaturation for 10 min at 95 °C; complementary base pairing for 15 min at 71.6 °C; quick drop to 4 °C; TProfessional Standard PCR Thermocycler, Core Life Sciences Inc., USA). Dibenzocyclooctyne-N-hydroxysuccinimidy ester (DBCO-NHS ester) was dissolved in DMSO to a concentration of 10 mM, added in a 100-fold molar excess to previously hybridized DNA, and incubated over night at room temperature. DBCO-functionalized dsDNA was purified via ethanol precipitation. To introduce an Azide group, Amino-Phalloidin (**((R)-4-Hydroxy-4-methyl-Orn^7)-Phalloidin; Bachem, Germany**) was pre-functionalised with Azidopropionic Acid Sulfo-N-hydroxysuccinimidyl (NHS) ester (Jena Bioscience, Germany). Therefore, Azidopropionic Acid Sulfo-NHS ester was dissolved in DMSO to a concentration of 10 mM, mixed in equal molar amounts with Amino-Phalloidin in 100 mM KH_2PO_4 pH 7.2, and incubated over night at room temperature. No further purifications were performed. Azide-containing peptides (Tab. S2; LifeAct®, Peptide Specialty Laboratories GmbH, Germany; pre-functionalized Azide-Phalloidin) were added in a 50 to 100-fold molar excess to DBCO-DNA and incubated over night at room temperature. To remove excess peptides, samples were Amicon® filtered (Amicon Ultra-4, PLGC Ultracel-PL Membrane, 10 kDa, Merck Millipore,
Germany) in 1x DBPS (w/o Calcium, w/o Magnesium; Thermo Fisher Scientific Inc., USA). The successful synthesis and the purity of the samples was verified with a native polyacrylamide gel (PAGE) (Fig. S1). Synthetic crosslinkers were stored at -20°C with no detectable degradation. They were added at different concentrations ranging from 0.24 nM to 9.6 µM (R = 0.0001 to 0.4).

Since it is known that phalloidin itself influences actin filaments by doubling their persistence length\[39\], we verified that according effects are insignificant compared to crosslinking effects (Fig. S3). This comparison illustrates that the physical connection between the binding domains is the crucial property necessary to induce the observed dynamics and the rich structural/mechanical phase space.

**EcoRV-HF®-digestion of sPX:** To investigate the necessity of two actin binding domains on one crosslinker for the stiffening of reconstituted actin networks, we performed bulk rheology measurements on actin with functional, undigested as well as EcoRV-HF®-digested sPX (incubated for 1 h at 37 °C). Crosslinker DNA was designed with an EcoRV digestion site almost in the middle of the double strand. A digestion of this results in a 32bp and a 28bp fragment. Prior to rheology measurements, 1624 ng sPX (final concentration of 0.24 µM in 175 µl final sample volume; R=0.01) were incubated with 5 µl of EcoRV-HF® (20000 Units/ml, New England Biolabs Inc., USA) and 1.75 µl of 10x CutSmart® buffer in a final volume of 20 µl for 1 h at 37 °C. No inactivation of the enzyme was performed. Corresponding PAGE analysis showed a digestion rate of at least 80 % after 1 h of incubation (Fig. S2). Additionally, we incubated double stranded control DNA without an EcoRV digestion site. We observed no detectable digestion of control DNA due to sequence specificity of the enzyme (data not shown). After EcoRV-HF®-incubation, the digested sample was directly added to actin, 10x G-buffer and 20x KME-buffer to a final volume of
175 µl. Thereby, actin polymerization was induced and rheological measurements were performed.

**Rheology:** 175 µl of sample was loaded to the dynamic shear rheometer (ARES, TA Instruments, USA) equipped with a cone (diameter 25 mm, 0.04 rad). The network between cone and plate was surrounded with a 2.5 ml 1x F-buffer bath similarly as described previously\cite{40,41} to avoid a direct contact of the sample with air. The sample chamber was sealed with a cap equipped with wet sponges to suppress evaporation. Measurements were performed at 20 °C and followed the sequence: (i) The time evolution of the polymerization was monitored for 2 h (one data point per minute; $\gamma = 5%$; $f = 1$Hz), which was subsequently followed by a (ii) short $f$ sweep ($\gamma = 5%$; $f = 0.01$ Hz to 30 Hz; 5 data points per decade), (iii) long $f$ sweep ($\gamma = 5%$; $f = 0.001$ Hz to 30 Hz; 21 data points per decade), (iv) short $f$ sweep, (v) $\gamma$ sweep ($f = 1$ Hz; $\gamma = 0.0125%$ to 100%; 20 data points per decade), (vi) short $f$ sweep, and (vii) $\gamma$ sweep. The sequence has been designed to test the robustness of the system over time and the frequency of 1 Hz has been chosen to display the values for $G_0$.

**Light scattering:** Static light scattering (Malvern Instruments Ltd., Zetasizer Nano ZSP, UK) was used to observe the dependence of actin morphology\cite{14,42} on synthetic crosslinker concentrations. The final actin concentration was 24 µM. The crosslinker concentration ranged from 0 µM to 9.6 µM ($R = 0$ to 0.4). The scattering of the sample was measured every minute for 1.5 h and scattering intensities were arithmetically averaged after 30 min of equilibration.

**Spinning Disk Confocal Microscopy:** For visualization, monomeric actin was mixed at a molar ratio of 3:1 with Phalloidin-TRITC purchased from Sigma-Aldrich. Synthetic crosslinkers with two LifeAct$^{\text{®}}$ binding domains (wLX) were added to yield a final $R = 0.333,$
R = 0.01, and R = 0.001 or with two Phalloidin binding domains (sPX) to yield a final R = 1, R = 0.1, and R = 0.01, respectively. Polymerization was initialized by increasing the salt concentration to 1x F-Buffer conditions after mixing all components with a final actin concentration of 3 µM. Immediately after starting the polymerization process the premixed solution was deposited into a sample chamber as described previously\textsuperscript{[11]}. Measurements were performed on a spinning disc confocal microscope (inverted Axio Observer.Z1/Yokogawa CSU-X1A 5000 (Carl Zeiss Microscopy GmbH, Germany), 100x oil immersion objective (Plan-Apochromat 100x/1.40 Oil DIC M27)) and recorded with a Hamamatsu Camera at an exposure time of 50 ms.

*Macroscopic behavior of actin in an inclined cuvette:* Three different samples have been prepared similarly as described above and 150 µl of final sample solutions have been pipetted into a small cuvette (1\textsuperscript{st} sample: 24 µM actin; 2\textsuperscript{nd} sample: 24 µM actin with synthetic crosslinkers featuring two phalloidin binding domains (sPX) at R = 0.4; 3\textsuperscript{rd} sample: 24 µM actin with sPX at R = 0.4, which were incubated for 1 h at 37 °C with 300 Units of EcoRV-HF\textsuperscript{®} (New England Biolabs Inc., USA)) prior to the experiment. A small amount of free Phalloidin-TRITC was added to dye the solution for a better contrast and visibility. After 40 min resting time, the cuvette with the respective sample was placed on an inclined plane with a 20 ° angle. Images have been recorded every second using a commercially available digital camera.
Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

Jessica Lorenz and Jörg Schnauß contributed equally to this work. We would like to thank Klaus Kroy, Manlio Tassieri, and Aftab Taiyab for fruitful discussions. We gratefully acknowledge funding by the Deutsche Forschungsgemeinschaft (DFG-1116/17-1), the European Research Council (ERC-741350) and the Fraunhofer Attract project 601 683.

Competing financial interests

The authors declare no conflict of interest.

Materials & Correspondence

Correspondence should be addressed to Jörg Schnauß & David M. Smith.
References


Supporting Information

Synthetic actin crosslinkers

Jessica S. Lorenz,1,3 Jörg Schnauß,1,2,3,* Martin Glaser,1,2 Martin Sajfutdinow,1, Carsten Schuldt,1,2 Josef A. Käs,2 David M. Smith1,*

1Fraunhofer Institute for Cell Therapy and Immunology (IZI), 04103 Leipzig, Germany
2Leipzig University, Peter Debye Institute for Soft Matter Physics, 04103 Leipzig, Germany
3These authors contributed equally to this work.
* Jörg Schnauß & David M. Smith

I Materials and Methods

S1. Actin preparation. Globular actin (G-actin) was prepared from rabbit muscle as described previously.[38] Actin was polymerised by the addition of 8.75 µl 20x F-Buffer (100 mM Tris-HCl pH 7.8, 2 mM CaCl₂, 2 M KCl, 20 mM MgCl₂, 4 mM ATP, 20 mM DTT, 0.2 % NaN₃) at a final concentration of 24 µM and a volume of 175 µl.

S2. Synthetic actin crosslinkers. Lyophilised oligonucleotides (Table S1; biomers.net GmbH, Germany) were resuspended in Millipore water and concentrations were spectrophotometrically determined by a NanoDrop 1000 (Thermo Fisher Scientific Inc., USA). Complementary oligonucleotides were hybridised in 100 mM KH₂PO₄ pH 7.2 at a final concentration of 25 mM in a thermocycler (denaturation for 10 min at 95 °C; complementary base pairing for 15 min at 71.6 °C; quick drop to 4 °C; TProfessional Standard PCR Thermocycler, Core Life Sciences Inc., USA). Dibenzocyclooctyne-N-hydroxysuccinimidy l ester (DBCO-NHS ester) was dissolved in DMSO to a concentration of 10 mM, added in a 100-fold molar excess to previously hybridised DNA, and incubated overnight at room temperature. DBCO-functionalised dsDNA was purified via ethanol precipitation. To introduce an Azide group, Amino-Phalloidin (((R)-4-Hydroxy-4-methyl-
Orn²⁻)-Phalloidin; Bachem, Germany) was pre-functionalised with Azidopropionic Acid Sulfo-N-hydroxysuccinimidyl (NHS) ester (Jena Bioscience, Germany). Therefore, Azidopropionic Acid Sulfo-NHS ester was dissolved in DMSO to a concentration of 10 mM, mixed in equal molar amounts with Amino-Phalloidin in 100 mM KH₂PO₄ pH 7.2, and incubated over night at room temperature. No further purifications were performed. Azide-containing peptides (Tab. S2; LifeAct®, Peptide Specialty Laboratories GmbH, Germany; pre-functionalized Azide-Phalloidin) were added in a 50 to 100-fold molar excess to DBCO-DNA and incubated over night at room temperature. To remove excess peptides, samples were Amicon® filtered (Amicon Ultra-4, PLGC Ultracel-PL Membrane, 10 kDa, Merck Millipore, Germany) in 1x DBPS (w/o Calcium, w/o Magnesium; Thermo Fisher Scientific Inc., USA).

The successful synthesis and the purity of the samples was verified with a native polyacrylamide gel (PAGE) (Fig. S1). Synthetic crosslinkers were stored at -20°C with no detectable degradation. They were added at different concentrations ranging from 0.24 nM to 9.6 µM (R = 0.0001 to 0.4).

Since it is known that phalloidin itself influences actin filaments by doubling their persistence length[39], we verified that according effects are insignificant compared to crosslinking effects (Fig. S3). This comparison illustrates that the physical connection between the binding domains is the crucial property necessary to induce the observed dynamics and the rich structural/mechanical phase space.

**S3. EcoRV-HF®-digestion of sPX.** To investigate the necessity of two actin binding domains on one crosslinker for the stiffening of reconstituted actin networks, we performed bulk rheology measurements on actin with functional, undigested as well as EcoRV-HF®-digested sPX (incubated for 1 h at 37 °C). Crosslinker DNA was designed with an EcoRV digestion site almost in the middle of the double strand. A digestion of this results in a 32bp and a 28bp fragment. Prior to rheology measurements, 1624 ng sPX (final concentration of 0.24 µM in
175 µl final sample volume) were incubated with 5 µl of EcoRV-HF\textsuperscript{®} (20000 Units/ml, New England Biolabs Inc., USA) and 1.75 µl of 10x CutSmart\textsuperscript{®} buffer (500 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 100 µg/ml BSA, pH 7.9) in a final volume of 20 µl for 1 h at 37 °C. No inactivation of the enzyme was performed. Corresponding PAGE analysis showed a digestion rate of at least 80 % after 1 h of incubation (Fig. S2). Additionally, we incubated double stranded control DNA without an EcoRV digestion site. We observed no detectable digestion of control DNA due to sequence specificity of the enzyme (data not shown). After EcoRV-HF\textsuperscript{®}-incubation, the digested sample was directly added to actin, 10x G-buffer (50 mM Tris-HCl pH 7.8, 1 mM CaCl\textsubscript{2}, 2 mM ATP, 10 mM DTT, 0.1% NaN\textsubscript{3}) and 20x KME-buffer (2 M KCl pH 7.8, 20 mM MgCl\textsubscript{2}, 4 mM EGTA) to a final volume of 175 µl. Thereby, actin polymerisation was induced and rheological measurements were performed.

**S4. Rheology.** 175 µl of sample was loaded to the dynamic shear rheometer (ARES, TA Instruments, USA) equipped with a cone (diameter 25 mm, 0.04 rad). The network between cone and plate was surrounded with a 2.5 ml 1x F-buffer bath (10 mM Tris-HCl pH 7.8, 100 mM KCl, 1 mM MgCl\textsubscript{2}, 0.2 mM ATP, 1 mM DTT) similarly as described previously\textsuperscript{[40,41]} to avoid a direct contact of the sample with air. The sample chamber was sealed with a cap equipped with wet sponges to suppress evaporation. Measurements were performed at 20 °C and followed the sequence: (i) The time evolution of the polymerisation was monitored for 2 h (one data point per minute; $\gamma = 5\%; f = 1$Hz), which was subsequently followed by a (ii) short $f$ sweep ($\gamma = 5\%; f = 0.01$ Hz to 30 Hz; 5 data points per decade), (iii) long $f$ sweep ($\gamma = 5\%; f = 0.001$ Hz to 30 Hz; 21 data points per decade), (iv) short $f$ sweep, (v) $\gamma$ sweep ($f = 1$ Hz; $\gamma = 0.0125\%$ to 100%; 20 data points per decade), (vi) short $f$ sweep, and (vii) $\gamma$ sweep (for more details see Supplemental Material sec. I). The sequence has been
designed to test the robustness of the system over time and the frequency of 1 Hz has been chosen to display the values for $G_0$.

**S5. Light scattering.** Static light scattering (Malvern Instruments Ltd., Zetasizer Nano ZSP, UK) was used to observe the dependence of actin morphology$^{[14,42]}$ on synthetic crosslinker concentrations. The scattering of the incident He-Ne-Laser beam (633 nm, 10 mW) by the sample (20 µl) in a quartz cuvette (Malvern, ZEN2112) was detected at a fixed angle of 173 °. To prevent evaporation during long term measurements, the cuvette was sealed with mineral oil (molecular biological grade, BioReagent, Sigma Aldrich Co., Germany). The final actin concentration was 24 µM. The crosslinker concentration ranged from 0 µM to 9.6 µM ($R = 0$ to 0.4). The scattering of the sample was measured every minute for 1.5 h and scattering intensities were arithmetically averaged after 30 min of equilibration.

**S6. Spinning Disk Confocal Microscopy.** Monomeric actin was mixed under 1x G-buffer conditions (5 mM Tris-HCl pH 7.8, 0.1 mM CaCl$_2$, 0.2 mM ATP, 1.0 mM DTT, 0.01 % NaN$_3$) at a molar ratio of 3:1 with Phallloidin-Tetramethylrhodamine B isothiocyanate (Phallloidin-TRITC, - Sigma-Aldrich Co., Germany) for visualisation. Synthetic crosslinkers with two LifeAct® binding domains (wLX) were added to yield a final $R = 0.333$, $R = 0.01$, and $R = 0.001$ or with two Phalloidin binding domains (sPX) to yield a final $R = 1$, $R = 0.1$, and $R = 0.01$, respectively. Polymerisation was initialised by increasing the salt concentration to 1x F-Buffer conditions (5 mM Tris-HCl pH 7.8, 0.1 mM CaCl$_2$, 0.1 M KCl, 1mM MgCl$_2$, 0.2 mM ATP, 1 mM DTT, 0.01 % NaN$_3$,4 mM EGTA) after mixing all components, leading to a final actin concentration of 3 µM. Immediately after starting the polymerisation process the premixed solution was deposited into a sample chamber as described previously$^{[11]}$. Measurements were performed on a spinning disc confocal microscope (inverted Axio Observer.Z1/Yokogawa CSU-X1A 5000 (Carl Zeiss Microscopy GmbH, Germany), 100x oil
immersion objective (Plan-Apochromat 100x/1.40 Oil DIC M27)) and recorded with a Hamamatsu Camera at an exposure time of 50 ms. Image series were exported using the ZEN 2 software provided by the company (Carl Zeiss Microscopy GmbH).

**S7. Macroscopic behaviour of actin in an inclined cuvette.** Three different samples have been prepared similar as described above and 150 µl of final sample solution have been pipetted into a small cuvette (1<sup>st</sup> sample: 24 µM actin; 2<sup>nd</sup> sample: 24 µM actin with synthetic crosslinkers featuring two phalloidin binding domains (sPX) at R = 0.4; 3<sup>rd</sup> sample: 24 µM actin with sPX at R = 0.4, which were incubated for 1 h at 37 °C with 300 Units of EcoRV-HF<sup>®</sup> (New England Biolabs Inc., USA) prior to the experiment. A small amount of free Phalloidin-TRITC was added to dye the solution for a better contrast and visibility. After 40 min resting time, the cuvette with the respective sample was placed on an inclined plane with a 20 ° angle. Images have been recorded every second using a commercially available digital camera.
II Synthesis of actin crosslinkers

Samples of the synthesis process were run on a 10 % (v/v) native polyacrylamide gel (PAGE) and further stained with SYBR® Gold Nucleic Acid Gel Strain (Thermo Fisher Scientific Inc., USA).

![Native PAGE analysis of the production of synthetic actin crosslinkers.](image)

**Figure S 1.** Native PAGE analysis of the production of synthetic actin crosslinkers. The engineering process of synthetic actin crosslinkers was stepwise monitored via 10 % (v/v) native polyacrylamide gel electrophoresis (PAGE) and visualised by SYBR® Gold Nucleic Acid Gel Strain staining. M, GeneRuler Low Range DNA Ladder; 1, 60bp hybridised DNA; 2, DBCO-functionalised 60bp DNA; 3, 60bp wLX; 4, 60bp sPX.

**Table S1.** Oligonucleotide sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5´→3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 fw NH2</td>
<td>NH2-GCTTTCTTCTCTTCAATACATCTTCTCACGTCGATATCACCATAA CTCAGGTAAAGGAGGTCAA</td>
</tr>
<tr>
<td>60 rv NH2</td>
<td>NH2-TCACCTCTTACCTGAGTTATGGTGATATCGACGTGAAGA TGTATTTAGAGAAGAAAGC</td>
</tr>
</tbody>
</table>

*bolded sequences are highlighting an EcoRV digestion site

**Table S2.** Peptide sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (N→C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azide-LifeAct®</td>
<td>Ac-MGVADLIKKFESISKEGG-azidolysine-amid</td>
</tr>
<tr>
<td>Amino-Phalloidin</td>
<td>Cyclo(-Ala-D-Thr-Cys-cis-Hyp-Ala-Trp-(4R)-4-hydroxy-4-Me-Orn) (Sulfide bond between Cys and indol-2-yl)</td>
</tr>
</tbody>
</table>
### III EcoRV-HF<sup>®</sup>-digestion of phalloidin crosslinkers (sPX)

We employed native PAGE analysis to verify that crosslinkers were cleaved and to derive the efficiency of the digestion for conditions used for incubations prior to rheology measurements and cuvette experiments displayed in Figure 5 in the main text.

**Figure S 2.** Native PAGE analysis of EcoRV-HF<sup>®</sup> digestion of 60bp sPX. **a,** The digestion of sPX through EcoRV-HF<sup>®</sup> was analysed via 10 % (v/v) native polyacrylamide gel electrophoresis (PAGE) and visualised by SYBR<sup>®</sup> Gold Nucleic Acid Gel Strain staining. M, GeneRuler Low Range DNA Ladder; 1, sPX w/o EcoRV-HF<sup>®</sup> incubation; 2, sPX incubated for 1 h at 37 °C with EcoRV-HF<sup>®</sup> according to manufactures manual. **b,** Band intensities were analysed using ImageJ. Roughly 80 % of the 60bp crosslinkers were cleaved by EcoRV-HF<sup>®</sup> after 1 h.
IV Influence of phalloidin

It is known that phalloidin binds to actin filaments and alters their mechanical properties by approximately doubling their persistence length.\textsuperscript{[39]} To show that the reported effects are indeed based on the crosslinking mechanism and not on the alteration of the filaments’ persistence length, we tested the mechanical behavior of actin networks enriched with different concentrations of phalloidin. These concentrations refer to crosslinker/actin ratios of $R = 0.001, 0.01, 0.1$ since each crosslinker carries two phalloidin binding domains.

With increasing phalloidin concentration the elasticity of the network slightly drops (Figure S3) in contrast to the concentration-dependent stiffening effects induced by sPX (Figure 4 in the main text). This illustrates that it is indeed the physical connection between the binding domains and the crosslinking causing the reported effects. Interestingly, an increase of $G'$ for stiffer filaments was observed for networks of DNA-nanotubes\textsuperscript{[43–46]}, however, here the fundamental structures/architecture were not altered as in the case of phalloidin binding to actin.
Figure S3. Phalloidin has a minor influence on network elasticity. To investigate the effect of phalloidin (peptides not connected via DNA template), we evaluated the impact of different phalloidin concentrations on the elasticity of the network. Employed concentrations refer to crosslinker/actin ratios of $R = 0.001, 0.01, 0.1$. In contrast to the sPX, no stiffening effects were observed and the network elasticity even slightly decreased. This behaviour proves that the physical connection between the binding domains is the crucial property for the sPX to induce the observed dynamics and the rich structural/mechanical phase space.
V Concentration dependency of the loss modulus G’’

Figure S4. Frequency sweeps for different wLx concentrations. Shown are frequency sweeps and according values for G’ and G’’, respectively, for three different wLx concentrations referring to the weakly crosslinked regime (R = 0.001 = 0.024 μM), the rheological fingerprint of the onset of bundle formation (R = 0.01 = 0.24 μM), and bundle network regime (R = 0.25 = 9.6 μM).
VI Concentration dependency of the loss modulus G''

Figure S 5. Loss modulus for the different crosslinkers at different concentrations. Different concentrations of the crosslinker induced only minor changes to the loss modulus G'' compared to their influence on the elastic modulus G' (see Figure 4 in the main text). Exceptions become apparent at high sPX concentrations, which induce the elasticity burst and thus out-of-equilibrium, glass-like dynamics.
VII Concentration dependent strain sweeps

Figure S 6. Strain sweeps. With increasing crosslinker concentration (shades of red) strain dependent stiffening of the network seems to evolve before strain yielding effects become more pronounced.
VIII Elasticity burst – decay times

Table S3. Decay constants of elasticity burst

<table>
<thead>
<tr>
<th>Sample</th>
<th>Decay constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 µM actin &amp; 2.4 µM sPX</td>
<td>221 s</td>
</tr>
<tr>
<td>24 µM actin &amp; 4.8 µM sPX</td>
<td>971 s</td>
</tr>
<tr>
<td>24 µM actin &amp; 2.4 µM fascin[14]</td>
<td>186 s</td>
</tr>
<tr>
<td>9.5 µM actin &amp; 0.95 µM fascin[22]</td>
<td>7560 s</td>
</tr>
</tbody>
</table>

The decay times of the elasticity bursts induced by sPX and fascin compare very well for same actin as well as crosslinker concentrations. Lower actin and/or crosslinker concentrations induce longer decay times.
**IX Elasticity burst with commercially purchased fascin**

**Figure S 7.** Elasticity burst and decay with natural fascin crosslinker. Time sweeps of the elastic modulus $G'$ are shown for two representative ratios of the biological strong actin-binding crosslinker fascin (solid curves are spline fits of the actual data points x). At the higher concentration (purple, $2.4 \mu M$, $R = 0.1$), the characteristic elasticity burst and subsequent decay are seen, similarly to synthetic sPx shown in this work, as well as previous reports with fascin under similar conditions. The evidently longer decay time compared to the same concentrations of sPx and fascin is attributed to decreased binding activity of the commercially produced protein. The lower concentration (blue, $0.24 \mu M$, $R = 0.01$), falls below the threshold for the elasticity burst behaviour, as is the case with the same concentration of sPx.
**Visualisation of actin network morphology**

As shown in Figure 4 in the main text and in accordance to previously published work\textsuperscript{32,47}, the morphology of actin networks is heavily influenced by the ratio of actin filaments and the involved crosslinkers. While for low crosslinker concentrations, the network is still isotropic as in the case of purely entangled actin filaments, for higher concentrations the formation of bundles can be clearly observed. This behaviour appears for both, the sPX and the wLX (Movie S1-S6), but is visually more pronounced for the wLX since transient crosslinkers develop longer and thicker bundles\textsuperscript{11,32}. I.e., the sPX form a network with smaller bundles, which appear less pronounced compared to wLX induced bundles display in Figure 4 in the main text (Figure S 8a). However, looking at the time depended behaviour of structures formed for differing sPX concentrations, it is clearly visible that for high crosslinker ratios the network is mostly comprised of static, relatively stiff bundles, which rarely fluctuate. For lower concentrations, the structures appear less stiff and fluctuate more with decreasing sPX concentrations emphasizing that less and/or softer bundles were formed (Movie S1-S3). To quantify this stiffening effect as an indicator for more pronounced bundle formation, an image correlation has been performed illustrating that networks are more static for higher sPX concentrations (Figure S 8b). Correlations of the image series were analysed using an in-house written MATLAB code.
Figure S 8. Actin network morphology. a, Three representative images illustrating the structural phases for different sPX concentrations (Left: R = 0.01 , Middle: R = 0.1 , Right: R = 1). b, The correlation factors for each example are shown above with differing lag time. The higher the sPX concentration the higher the correlation of the subsequent pictures, which illustrates that the underlying structures are stiffer and thermally fluctuate less.
XI Supplementary Movies

All Movies are shown in 0.5x real-time.

**Supplementary Movie 1:** Actin network crosslinked with sPX at $R = 0.01$

A time series of a Rhodamine-Phalloidin stabilised actin network at 3 $\mu$M in a weakly crosslinked regime (sPX at $R = 0.01$). Actin filaments or arrangements of several filaments fluctuate in a non-percolated network.

**Supplementary Movie 2:** Actin network crosslinked with sPX at $R = 0.1$

A time series of a Rhodamine-Phalloidin stabilised actin network at 3 $\mu$M in an intermediate crosslinked regime (sPX at $R = 0.1$). Rigid arrangements of several actin filaments and small actin bundles fluctuate less in a semi-percolated network.

**Supplementary Movie 3:** Actin network crosslinked with sPX at $R = 1$

A time series of a Rhodamine-Phalloidin stabilised actin network at 3 $\mu$M in a highly crosslinked regime (sPX at $R = 1$). Short rigid actin bundles hardly fluctuate within the percolated network.

**Supplementary Movie 4:** Actin network crosslinked with wLX at $R = 0.001$

A time series of a Rhodamine-Phalloidin stabilised actin network at 3 $\mu$M in a weakly crosslinked regime (wLX at $R = 0.001$). Actin filaments fluctuate freely in a weakly crosslinked network.
**Supplementary Movie 5:** Actin network crosslinked with wLX at R = 0.01

A time series of a Rhodamine-Phalloidin stabilised actin network at 3 µM in an intermediate crosslinked regime (wLX at R = 0.01). A coexistence regime with freely fluctuating actin filaments in the presence of long, thick actin bundles occurs.

**Supplementary Movie 6:** Actin network crosslinked with wLX at R = 0.333

Z-stack of a Rhodamine-Phalloidin stabilised actin network at 3 µM in a highly crosslinked regime (wLX at R = 0.333). Long, thick, and ridged actin bundles hardly fluctuate within the percolated bundle network.